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MicroRNAs: mediators of EGFR targeted therapy resistance in renal cell carcinoma

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MicroRNAs: mediators of EGFR targeted therapy resistance in renal cell carcinoma

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Abbreviations

A

ABC	ATP-binding cassette
ABCB1	ATP binding cassette subfamily B member 1
ABCG2	ATP binding cassette subfamily G member 2
AGO	Argonaute
AIB1	Amplified in breast cancer 1
AKT	Protein kinase B
AP1	Activator protein 1

B

B2M	Beta-2-microglobulin

C

ccRCC	clear-cell renal cell carcinoma
CDK	Cyclin-dependent kinases
cDNA	Complementary deoxyribonucleic acid
CRC	Colorectal cancer
CXCR-4	C-X-C chemokine receptor type 4

D

DGRC8	Digeorge syndrome critical region gene 8
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol

E

EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial–mesenchymal transition
EPO	Erithropoietin
ERK	Extracellular signal-regulated kinase

F

FBS	Fetal bovine serum
FISH	Fluorescent In Situ Hybridization

G

GEMIN-3	Gem-associated protein 3
GLB	Glo lysis buffer
GLUT1	Glucose transporter 1

H

HER	Human epidermal growth factor receptor
HIF	Hypoxia-inducible factor

K

KRAS	Kirsten rat sarcoma viral oncogene homolog

M

MAPK	Mitogen-activated protein kinase
MAX	Myc associated factor X
MDR1	Multidrug resistance protein 1
mRNA	messenger Ribonucleic acid
microRNA	micro Ribonucleic acid
MNT	Max-binding protein
mRCC	metastatic renal cell carcinoma
mTOR	mammalian target of rapamycin

N

NF-κB	Factor nuclear kappa B
NSCLC	Non-small cell lung cancer

P

PDGF-β	Platelet-derived growth factor β
PI3K	Phosphatidylinositide 3-kinase
PTEN	Phosphatase and tensin homolog

R

RAG2	Recombination activating gene 2
RAR-α	Retinoic acid receptor
RCC	Renal cell carcinoma
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid

S

SEM	Standard error of the mean
SP1	Specificity protein 1
STAT3	Signal transducer and activator of transcription 3

T

TBS	Tris-buffered saline
TGF-α	Transforming growth factor α
TIMP-3	Tissue inhibitor of metalloproteinase 3
TKI	Tyrosine kinase inhibitors
TRBP	TAR RNA binding protein

TRPS1	Trichorhinophalangeal syndrome I

U

UTR	Untranslated region

V

VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VHL	Von Hippel-Lindau

W

WHO	World Health Organization

X

XPO5	Exportin 5

Resumo

O carcinoma de células renais de células claras (CCRcc) é o subtipo mais frequente de CCR e está associado à perda ou inativação do gene *VHL*, que resulta, consequentemente, numa acumulação do fator de transcrição associado à hipoxia (HIF) em condições de normóxia. A acumulação deste fator potencia a transcrição de genes como o TGF-α e VEGF, que, em última instância, promovem a ativação de vias de sinalização proliferativas e angiogénicas centrais na progressão do CCRcc. O crescente conhecimento das vias de sinalização envolvidas no desenvolvimento e progressão do CCR permitiu, nos últimos anos, o desenvolvimento de novas terapias dirigidas a alvos moleculares específicos, com o intuito de melhorar as opções terapêuticas e a eficiência das mesmas. Um desses agentes é o bevacizumab, um inibidor de angiogénese, já usado no tratamento de doentes com CCR. Adicionalmente, o erlotinib, um inibidor do receptor de fator de crescimento epidérmico (EGFR), pode-se revelar uma potencial opção terapêutica no CCR dada a sobreexpressão do EGFR e sobreativação de vias associadas, verificadas neste modelo tumoral. Contudo, o desenvolvimento de resistência a terapias dirigidas é altamente frequente em CCR, o que enfatiza a importância de estudar mecanismos de escape à mesma. Os microRNAs (miRNAs), uma família de pequenas moléculas de RNA não codificantes, têm vindo a ser associados como importantes mediadores de resistência à terapia, visto que regulam a expressão genética e, consequentemente, diversos processos biológicos. A sobreexpressão do miRNA-21 tem vindo a ser associada com a resistência a terapias dirigidas ao EGFR, por ativação *downstream* da via PI3K/AKT. A base deste mecanismo de resistência assenta no facto deste miRNA regular a transcrição de PTEN, uma fosfatase com atividade supressora tumoral que regula negativamente a atividade de AKT. O miRNA-210, um miRNA induzido pelo HIF, também poderá estar associado a mecanismos de resistência a terapias dirigidas ao EGFR, ao inibir a transcrição do MNT. O MNT é um importante regulador do ciclo celular e inibidor transcricional que compete com c-Myc pela ligação a Max. Visto que o c-Myc é um fator de transcrição ativado por vias de sinalização associadas ao EGFR e importante impulsionador do avanço do ciclo celular quando ligado a Max, a desregulação desta interação poderá comprometer a resposta à terapia.

O presente estudo *in vitro* foi realizado com recurso a duas linhas celulares: uma linha celular renal tumoral, FG-2, e uma linha celular renal tumoral resistente ao erlotinib, FG-2R, estabelecida no decorrer do presente estudo. Ensaios de viabilidade celular realizados na

linha celular FG-2 demonstraram a eficácia, *in vitro*, do erlotinib na inibição da proliferação celular (P= 0.006). Adicionalmente, verificou-se um efeito sinérgico do bevacizumab e erlotinib no tratamento, *in vitro*, da linha FG-2 (P= 0.007). A combinação do erlotinib com bevacizumab pode-se revelar eficaz, visto que afetam diferentes receptores, EGFR e VEGFR, mas os quais possuem vias de sinalização associadas comuns, como a PI3K/AKT e a MAPK/ERK. Contudo, este efeito no controlo da proliferação celular não foi previamente observado em ensaios clínicos que recorreram ao uso do erlotinib isolado ou em combinação com bevacizumab em doentes com CCR metastático. Posteriormente, avaliou-se os níveis intracelulares e extracelulares do miRNA-21 e do miRNA-210 nas linhas celulares em estudo. Os níveis intracelulares e extracelulares do miRNA-21 não apresentaram variações na linha FG-2R quando comparado com a linha FG-2 (P= 0.185 e P= 0.319, respetivamente). No entanto, o miRNA-210 apresentou-se sobreexpresso tanto a nível intracelular (P= 0.009) como a nível extracelular (P= 0.017) na linha FG-2R quando comparado com a linha FG-2. Concomitantemente com a sobreexpressão deste miRNA, os níveis de mRNA do MNT, um alvo do microRNA-210, encontraram-se diminuídos na linha FG-2R (P< 0.001). No presente estudo foi possível também demonstrar que simultaneamente com a sobreexpressão de miRNA-210 e subexpressão de *MNT* na linha FG-2R, verifica-se um aumento da interação c-Myc-Max na fase G1 do ciclo celular quando comparado com a linha FG-2 (1.76 ± 0.34).

Em conclusão, o miRNA-210 poderá estar envolvido num mecanismo de resistência ao erlotinib por levar à diminuição da expressão de *MNT* e consequente aumento da interação de c-Myc-Max, promotora da entrada no ciclo celular e da proliferação celular. Porém, estudos adicionais serão necessários para validar o mecanismo de resistência proposto e para, possivelmente, vir a considerar este miRNA como indicador de resposta à terapia e até mesmo como um alvo terapêutico.

Abstract

Clear cell renal cell carcinoma (ccRCC) is the most frequent hystologic subtype of RCC and is associated with loss or inactivation of the *VHL* gene, which leads to the accumulation of the hypoxia-associated transcription factor (HIF) in normoxic conditions. HIF triggers the transcription of different genes, such as TGF-α and VEGF, ultimately promoting the activation of crucial proliferative and angiogenic signaling pathways in ccRCC progression. In recent years, the discovery of relevant signalling pathways to RCC initiation and progression has allowed the development of molecular targeted drugs that take advantage of genetic addictions, dependences and vulnerabilities of cancer cells in order to maximize treatment effectiveness. Bevacizumab, an angiogenesis inhibitor, is an example of drugs developed to target more specifically cancer cells addictions and is already used in RCC patient´s treatment. Additionally, erlotinib, an epidermal growth factor receptor (EGFR) inhibitor, could represent an effective therapeutic option in RCC treatment since EGFR upregulation and EGFR-related signaling pathways overactivation is already described in this tumor model. Treatment resistance is highly frequent in RCC revealing the necessity of unveiling resistance mechanisms. MicroRNAs (miRNAs), a family of small non-coding RNAs, have been reported as mediators of treatment resistance mechanisms, since they regulate genetic expression and, consequently different cellular processes. MiRNA-21 overexpression has been associated with EGFR-targeted therapies resistance, through PI3K/AKT downstream activation. The resistance mechanism is mainly through PTEN downregulation, a tumor-suppressing phosphatase that antagonizes the function of PI3K and negatively regulates AKT activity. MiRNA-210 could also be associated with EGFR targeted therapies resistance by downregulating *MNT*. MNT is an important cell cycle regulator due to its ability of competing with c-Myc for Max binding and, ultimately, due to its transcriptional inhibitor activity. Since c-myc is a transcription factor activated through EGFR-related signalling pathways and triggers cell cycle entry and progression when bound to Max, dysregulation of this interaction could lead to therapy resistance.

The present study was performed using two different cell lines: a tumoral renal cell line, FG-2, and an erlotinib-resistant tumoral renal cell line, FG-2R, established during the development of this project. Viability studies performed in the FG-2 cell line demonstrated the erlotinib effectiveness in inhibiting cellular proliferation (P= 0.006). Additionally, a synergic effect of bevacizumab and erlotinib was also demonstrated when treating the FG-2 cell line, *in vitro* (P= 0.007). The combination of erlotinib and bevacizumab may be

beneficial since they target different receptors, namely EGFR and VEGFR, that share downstream signaling pathways, such as PI3K/AKT and MAPK/ERK.The results obtained are not fully in line with the results of clinical trials performed in metastatic RCC patients, since the benefic effect of erlotinib alone or in combination with bevacizumab was hard to prove. Subsequently, miRNA-21 and miRNA-210 extracellular and intracellular levels were evaluated in the cell lines in study. On the one hand, intracellular and extracellular miRNA-21 levels were not altered in the FG-2R cell line when compared to the FG-2 cell line (P= 0.185 and P= 0.319, respectively). On the other hand, miRNA-210, which is regulated by HIF, presented higher intracellular (P= 0.009) and extracellular (P= 0.017) levels in the FG-2R cell line when compared with the FG-2 cell line. Concomitantly with miRNA-210 overexpression, MNT mRNA levels, a known target of this microRNA, were downregulated in the FG-2R cell line. *In vitro* studies performed demonstrated that simultaneously with miRNA-210 overexpression and *MNT* underexpression in the FG-2R cell line, c-Myc-Max interaction levels were higher in the G1 phase of the cell cycle when compared with the FG-2 cell line (1.76 ± 0.34).

In conclusion, miRNA-210 could mediate an erlotinib resistance mechanism by *MNT* downregulation and consequent increase in the c-Myc-Max interaction, promoting cell cycle entry and cell proliferation. However, additional studies are necessary to validate the proposed resistance mechanism and to, ultimately, consider this microRNA as a biomarker for treatment response and even as a therapeutic target.

1. Introduction

1.1 Cancer

Cancer has rose as an emergent public health problem, since it represents one of the leading causes of death worldwide [1, 2]. According to recent reports, an estimated 3,45 million new cancer cases and 1,75 million cancer-related deaths occurred in 2012 in Europe. Breast cancer, colorectal cancer, prostate cancer and lung cancer cases represent almost half of cancer cases, in this continent [3]. These same types of cancer are also associated with the most cancer-related deaths in Europe in 2012, although stomach cancer and pancreatic cancer, 4th and 5th respectively, represent more deaths than prostate cancer [3]. Many risk factors promote carcinogenesis such as: tobacco use, alcohol use, unhealthy diet and physical inactivity but ageing is considered the biggest one [1, 2]. The average life expectancy of the world population is increasing which, not only, promotes the accumulation of genetic mutations on somatic cells but also is associated with a weakening of the immune system, leading, ultimately, to a more suitable environment to tumor development [1].

Nowadays, cancer is considered a heterogeneous disease that develops trough interactions between environmental and genetic factors, involving the dysregulation of multiple pathways responsible for the fundamental cell processes, such as death, proliferation, differentiation and cell migration [1, 2]. Several evidences indicate that carcinogenesis is a multiphase process, which is associated with genetic and epigenetic modifications that promote a progressive transformation of normal cells into tumoral cells [4]. More in depth, carcinogenesis can be divided into three main phases: initiation, promotion and progression. Initiation is the first stage of tumor development where an irreversible genetic modification occurs through one or more point mutations, translocations, deletions, or even insertions in the DNA. Secondly, promotion is a reversible process that does not involve DNA structure modifications but is associated with a selective clonal expansion of initiated cells, producing a larger population of cells that are at risk of further genetic changes and malignant conversion. Ultimately, tumor progression can occur, characterized by the expression of a malignant phenotype and the tendency of malignant cells to acquire additional aggressive characteristics over time through further genetic and epigenetic modifications [5, 6]. During the carcinogenesis, genes that suffer modifications

and promote tumor initiation and progression are divided into three main groups: oncogenes, tumor suppressor genes and apoptosis-regulatory genes [7]. Proto-oncogenes are converted into oncogenes by gain-of-function mutations that may occur through three major mechanisms: point mutations, that result in a mutated and constitutively acting protein; gene amplification, that leads to protein overexpression; and chromosomal translocation, that alters transcription regulation of proto-oncogenes. These mutations act dominantly since a mutation in only one of the two alleles is sufficient for cancer induction. Tumor-suppressor genes commonly encode proteins related with cell proliferation inhibition. Only one copy of the tumor-suppressor genes is needed to maintain cell normal function and proliferation control, so loss of function of tumor-suppressor genes needs to occur in both alleles and most frequently involves point mutations in one allele and loss of the second allele by a deletion, recombinational event, or even chromosomal nondisjunction. Finally, apoptosis-regulatory genes can promote tumor development through both mechanisms [8]. This type of modifications are promoted through chemical, physical and biological carcinogens present in our daily life and also through endogenous factors [9].

Until the year 2000 there was no proper definition and characterization of tumoral cells that could differentiate them from somatic cells. Thus, Hanahan and Weinberg proposed the *Hallmarks* of cancer for rationalizing the complexities of neoplastic disease and allowing the needed differentiation [4]. First, six *Hallmarks* were suggested: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [4]. Since then more *Hallmarks* have been added to a more proper characterization of such a complex disease as cancer, such as evading immune destruction, metabolism dysregulation, tumor-promoting inflammation and also genetic instability and mutations, that promote tumor initiation and development [10]. The constant evolution in cancer scientific research resulted in the discovery of a large number of factors involved in tumor progression and also tumor cell vulnerabilities and dependencies, allowing the study and development of new therapeutic approaches.

1.2 Cancer Treatment

Chemotherapy, along with surgery and radiotherapy, has been a crucial approach for cancer treatment. These different types of treatment can be used alone or in different combinations, and either simultaneously or sequentially. However, chemotherapy/radiotherapy-induced cell damage occurs preferentially but not exclusively in cancer cells, causing several side-effects [11]. For this reason, nowadays, a big effort is being made in order to improve a personalized medicine that focus on the discovery and development of molecular targeted drugs that take advantage of genetic addictions, dependences and vulnerabilities of cancer cells. This type of methodology would be more specific than previous described approaches by minimizing side effects on normal cells [12]. Additionally, different high-throughput technologies, still under development, like genome sequencing and various kinds of microarrays, will allow the knowledge of the genetic, epigenetic and proteomic background, from each individual and tumor, which ultimately will lead to a more personalized treatment [13].

The concept of targeting a pathogenic driver abnormality using a small molecule was first validated in 1988 by the successful treatment of patients with acute promyelocytic leukemia harboring translocations in the *RARα* (retinoic acid receptor) gene with all-trans retinoic acid [14]. Additionally, the use of imatinib, a BCR-ABL inhibitor, as a chronic myeloid leukemia treatment in 1996, marked the era of the design of small therapeutic molecules applied in cancer treatment [15]. The 8-year estimated overall survival rate for patients with this malignancy, characterized by the BCR-ABL translocation, has improved from 42-65% in 1983-2000 to 87% since 2001, mainly due to the use of imatinib as initial therapy [16]**.**

Even with all the developments and improvements in cancer therapy, resistance to treatment still exists. Therapy failure is often due to development of drug resistance that may be inherent in a subpopulation of heterogeneous cancer cells or acquired subsequent to treatment [11]. A well characterized resistance mechanism is related with the activity of ABC transporters. ABC transporters are transmembrane proteins responsible for the transport of a wide variety of substrates across cellular membranes, including hydrophobic drugs and antibiotic [17, 18]. Overexpression of these proteins can be associated with reduced drug uptake, increased drug efflux and lead to lower drug efficacy and possibly to acquire drug

resistance due to low drug levels in the cytoplasm [18]. The major members of the ABC transporters associated with multidrug resistance in cancer cells are ABCB1/MDR1, ABCCs and ABCG2 [19]. Although, several have been identified as transporters of cancer chemotherapeutics agents, acquired chemotherapy drug resistance can occur at many levels, modulated either by genetic or epigenetic factors. In fact, recent data demonstrate that the activity of certain microRNAs (miRNAs) might be altered in order to achieve resistance to chemotherapy [12]. In the same line of thought, miRNAs can be as well linked to acquired resistance in molecular targeted therapy in several malignancy treatments.

1.3 MicroRNAs and Therapy Resistance

MiRNAs are a class of non-coding RNAs (19-25 nucleotides in length) that control gene expression by either degrading or blocking translation of mRNAs a process that depends on the miRNA-mRNA target degree of complementarity [20]. MiRNA biogenesis initiates at the nucleus with the transcription of a primary RNA, the pri-miRNA, by RNA polymerase II [21]. Afterwards, pri-miRNA is processed by Drosha, an RNase III endonuclease, alongside with cofactor DGCR8, creating a pre-miRNA [22]. The pre-miRNA is exported to the cytoplasm by the nuclear export protein XPO5, where it is further processed by DICER, leading to the production of a mature 22 nucleotide-stranded molecule [21]. The mature miRNA enters the RNA-induced silencing complex (RISC), whose main components are TRBP (Tar RNA-binding protein), AGO1-4 (Argonaute 1-4), GEMIN3 (Gem-associated protein 3) and GEMIN4 (Gem-associated protein 4) becoming, ultimately, a functional miRNA [23]. This complex binds to the target mRNA at 3´UTR region by complementarity leading to gene silencing (Figure 1) [24].

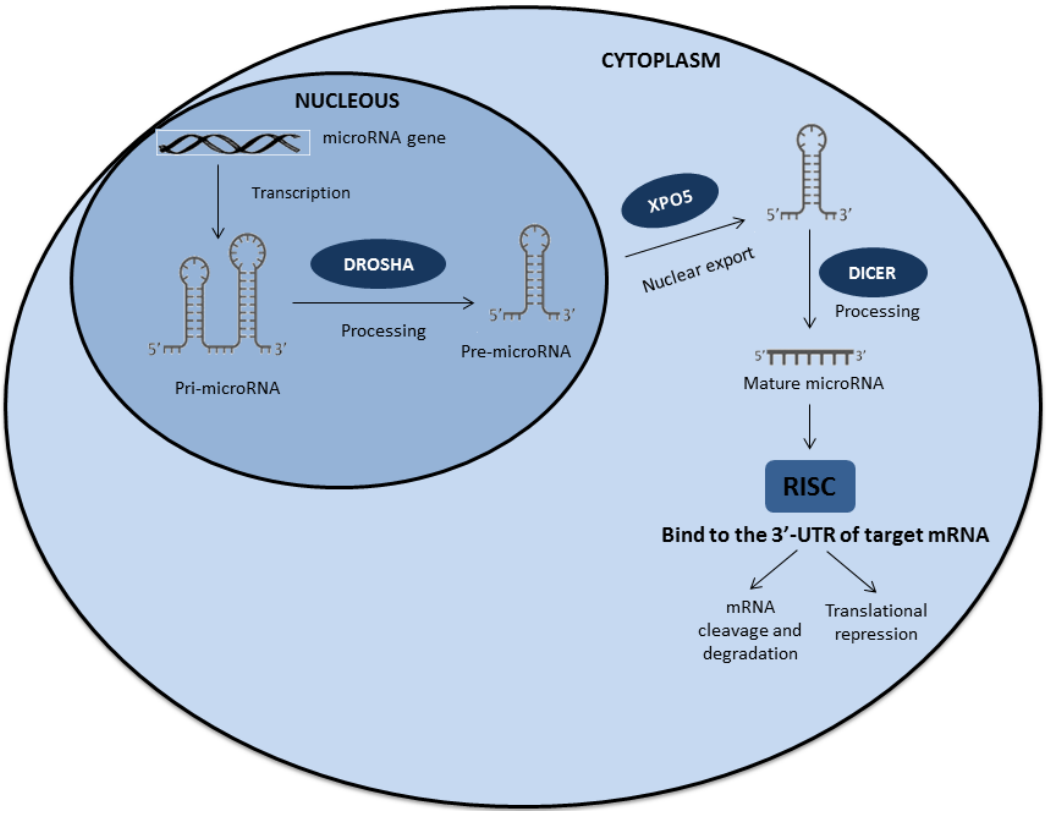

Figure 1- MicroRNAs biogenesis and regulation mechanisms (Adapted from Dias, F., et al. [32]).

MiRNA expression is dynamic, since a miRNA can regulate up to 100 different mRNAs and that more than 10.000 mRNAs appear to be directly regulated by miRNAs [25]. Thus, changes in the miRNA processing and expression patterns could be associated with different pathologies, including cancer, suggesting that miRNAs are involved in many cellular function disorders, which could ultimately lead to carcinogenesis [26].

After the discovery of miRNA-15a and miRNA-16-1 as the first miRNAs with tumor suppressor functions in chronic lymphocytic leukemia in 2002, several others miRNAs have been described as mediators of cancer related-signaling pathways, regulating proliferation, apoptosis, angiogenesis and even epithelial-mesenchymal transition (EMT), a key step for the metastatic process [27, 28]. Depending on mRNA targets and their impact on different biological processes, miRNAs can be described to act as oncogenes - oncomiRNAs, tumor suppressors – miRNAs tumor suppressors or even modulators of cancer stem cells and metastasis formation. OncomiRNAs are, usually, overexpressed in cancer since they are known to downregulate tumor suppressor genes and tumor suppressor miRNAs are responsible for downregulating oncogenes, so are mostly under-expressed in malign neoplasms [29, 30]. However, this dichotomous approach may have its limitations. For

example, miRNA-17 is associated, in B-cell lymphoma, with faster tumor development, while it can suppress cancer growth by downregulating AIB1 expression in breast cancer [31, 32]. Therefore, it is important to take into account that a microRNA may act as an oncomiRNA or a tumor suppressor miRNA depending on the type of tissue.

One of the most interesting features of miRNAs is that they are able to circulate in the majority of biological fluids [33]. Circulating miRNAs are highly stable even when submitted to severe conditions, such as boiling, very low or high pH, extended storage, and several freeze-thaw cycles, conditions that would normally degrade most RNAs [34]. They also exhibit protection from RNase activity due to the fact that most microRNAs circulate inside of exosomes in the different biological fluids [35]. Exosomes are membrane-bound vesicles of 40–100 nm in diameter released from most cell types, including cancer cells, enriched in cholesterol, sphingomyelin and ceramide as well as lipid raft associated proteins which allows them to be highly stable in circulation [36-38]. Due to its features, this type of extracellular vesicles has the ability of horizontal cargo transfer interacting with neighboring or distant cells [39]. In cancer, tumor-derived exosomes containing miRNAs can modulate biological processes inside of recipient cells, in a systemic manner [35].

Regarding the miRNAs role in cell communication and cellular processes such as proliferation, apoptosis or angiogenesis, it is important to take into account the effect that changes in miRNA levels may have in treatment response, since targeted therapies are used for specific proteins and signal pathways related with this biological processes [27, 28].

In fact, many studies have been identifying miRNAs as molecules involved in acquired resistance to targeted therapies in different tumor models [40]. For example, studies in non-small cell lung cancer (NSCLC) performed by Shen *et al.* stated that miRNA-21 correlated with PTEN levels, one of its targets, modulates gefitinib resistance [41, 42]. A significantly higher expression of miRNA-21 and a reduction in PTEN protein levels was found when comparing tumor tissues with adjacent normal tissues of patients with NSCLC. It was also described that patients with the highest levels of expression of miRNA-21 and lowest protein levels of PTEN exhibited poor tyrosine kinase inhibitors (TKI) clinical response and shorter overall survival [42]. Simultaneously, *in vitro* studies were performed using a PC-9 TKI sensitive cell line and a gefitinib-resistant cell line, PC-9/GR, in order to test the effect of high miRNA-21/low PTEN expression on modulation of TKI sensitivity. MiRNA-21 was upregulated concomitantly to downregulation of PTEN in PC-9/GR cells,

which leads to activation of AKT and ERK pathways and, ultimately, low sensitivity to gefitinib [42]. Ragusa and co-workers also demonstrated that miRNAs might be involved in targeted therapy acquired resistance in colorectal cancer (CRC). They performed an expression profile of a large number of miRNAs in two human colorectal cancer cell lines, one cetuximab sensitive (Caco-2) and other cetuximab resistant (HCT-116). MiRNAs let-7b and let-7e were downregulated in the cetuximab resistant cell line, HCT-116, in which signaling downstream of KRAS remains activated. Let-7 family members are known to target KRAS, so their downregulation could be a mechanism that contributes to cetuximab resistance [43] (Table 1). Regarding renal cell carcinoma (RCC), our tumor model of study, studies have not yet been performed relating microRNAs and targeted therapies resistance but this theme represents an interesting field of study since therapy resistance is a big issue in this type of cancer [44].

Cancer	Targeted Therapy	miRNA involved	Ref.
Head and neck cancer	Cetuximab	↓ miRNA-212	[33]
Lung cancer	Erlotinib	↑ miRNA-200 family	[34]
	Gefitinib	↑ miRNA-21	[35]
		↑ miRNA-30c, ↓ miRNA-103, ↓ miRNA-203, ↑ miRNA-221 and ↑ miRNA-222	[36]
		↑ miRNA-214	[37]
		↑ miRNA-374a and ↓ miRNA-548b	[38]
Gastric cancer	Trastuzumab	↑ miRNA-21	[39]
Breast cancer	Trastuzumab	↑ miRNA-21	[40]
		↑ miRNA-221	[41]
		↓ miRNA-375	[42]
	Lapatinib Neratinib Afatinib	↓ miRNA-630	[43]
Coloretal cancer	Cetuximab	↓ miRNA-let7b, ↓ miRNA-let7e and ↑ miRNA-17	[44]

↑: Upregulation; ↓: Downregulation.

Figure 2 - Summary of the miRNAs involved in the acquired resistance to ErbB family targeted therapies by cancer type.

1.4 Renal Cell Carcinoma

Kidney cancer represents a heterogeneous group of renal carcinomas with different molecular and histological patterns and a wide range of clinical characteristics and treatment responses [45].

The most common kidney solid cancer in adults is the renal cell carcinoma (RCC) accounting for approximately 90% of kidney neoplasms and representing 2-3% of all cancers worldwide [46, 47]. There is a 1.5:1 predominance of new RCC cases diagnosed in men over women, with a incidence peak between the age of 60 and 70 years old [47]. In Europe, RCC represents the 9th most incident adult malignancy (84400 new cases and 34700 kidney cancer-related deaths in 2012), being Portugal one of the European countries with the lowest incidence rates [3, 46, 47]. Incidence and mortality rates of RCC show a geographic variation: the highest incidence rates are observed in Northern America, Western Europe and Australia, whereas the lowest are observed in India, China and Africa. The highest mortality rates are registered in the European continent, mainly in the Central and Eastern countries, followed by the western and southern regions (Figure 2) [46, 47].

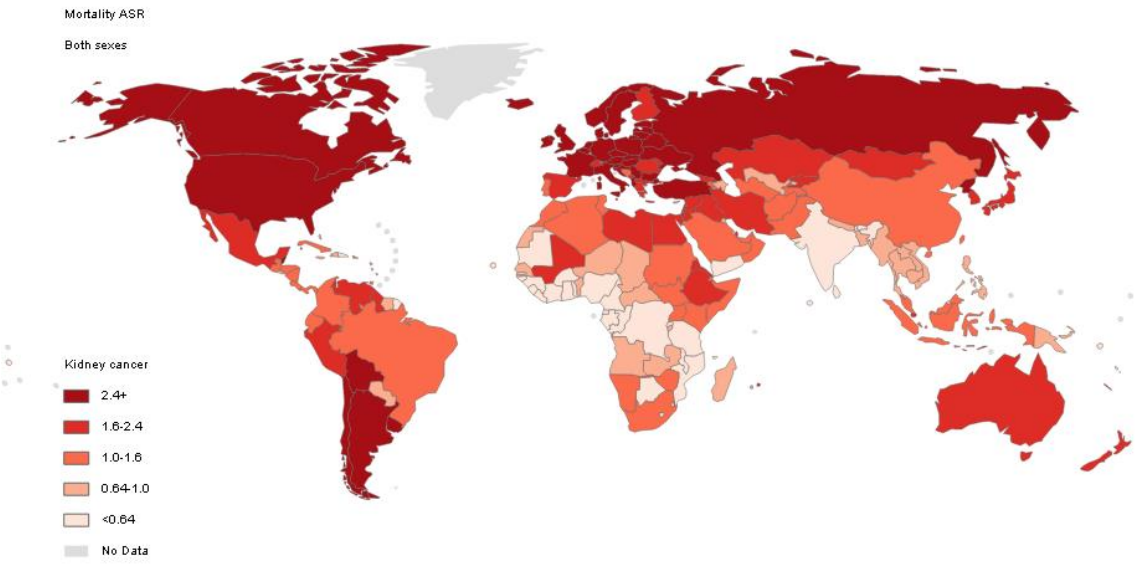

Figure 3 - Kidney cancer mortality rates per 100 000 habitants, in both genres (Adapted from GLOBOCAN 2012, IARC).

The geographic disparity observed in RCC incidence rates worldwide can be attributed to differences in diagnosis frequency, access to health care, genetic inheritance and prevalence of certain lifestyle habits and/or environmental risk factors. Different epidemiological studies revealed that smoking habits, obesity, hypertension and also acquired cystic disease and family history of RCC seem to be involved in the etiology and development of RCC [46, 48-51].

Currently, most RCCs are detected accidentally as a consequence of imaging examinations, such as computed tomography and magnetic resonance imaging [52]. This occurs due to the fact that many renal masses remain asymptomatic and nonpalpable until the late stages of the disease [52]. In case of accidentally diagnosis, the prognosis is good and further exams, such as biopsies and aspiration cytology, are needed to confirm the tumor neoplastic nature. Clinical symptoms, such as flank pain, gross hematuria, palpable abdominal mass, and paraneoplastic syndromes, only appear when the tumor presents a larger diameter, which can be associated with a worse prognosis [47, 53, 54]. Regarding this facts and the nonexistence of a standard screening test, one-third of patients are diagnosed with local invasive disease or metastatic disease [52].

The classification system most widely accepted for histologic grading of RCC is the Fuhrman nuclear grade, grouping the RCC cases into four main groups [53, 54]. Fuhrman nuclear grades 1-2 are associated with tumors in initial stages of development and better prognosis and 3-4 with worst prognosis due to a greater tumor malignancy and aggressiveness. [53, 54].

Therapeutics options in RCC depend on many factors such as: tumor size and location, local or distant invasiveness, renal function and others [47]. In initial stages of RCC development, surgical intervention is the primary treatment used. However, surgery does not represent a good therapeutic option, when used alone, in patients with metastatic disease [55, 56]. Due to the fact that RCC is highly resistant to cytotoxic chemotherapy and radiotherapy, until recently treatment options for patients with metastatic RCC (mRCC) were extremely limited [57, 58]. Cytokine therapies such as interleukin-2 and interferon-alpha were one of the first systemic treatment options in mRCC but they proved to be ineffective since only a small percentage of the patients showed benefit in long-term disease-free survival [59, 60]. More recently, due to the discovery of which molecular pathways are constitutively activated and are important for RCC development and progression, targeted agents such as receptor

tyrosine kinase inhibitors (TKIs), vascular endothelial growth factor (VEGF) antibodies, and mammalian target of rampamycin inhibitors (mTORs) started to represent a fundamental approach in mRCC treatment [52, 56, 61]. Although mRCC patient's outcome has improved, targeted therapies acquired resistance still occurs after a median of 5-11 months due to genetic and epigenetic changes but mainly through downstream activation of the signaling pathway targeted therapy site [40, 44, 62].

1.5 Clear Cell Renal Cell Carcinoma oncobiology and its relation with microRNAs

According to the ISUP Vancouver Modification of World Health Organization (WHO), there are more than twenty-four histologic subtypes of RCC being the clear cell, papillary (type I and II) and chromophobe the most frequent subtypes [63-65]. The clear cell renal cell carcinoma (ccRCC) is considered the most aggressive subtype [63]. ccRCC is associated with the *von Hippel-Lindau* syndrome, a hereditary condition, or with the loss of function of the tumor suppressor gene, *VHL*, in sporadic cancer cases [66, 67]. Usually inactivation of the *VHL* gene present in the chromosome 3p25.3 occurs through a mutation in one of the alleles and a deletion on the second one [66]. In fact, in 90% of the ccRCC cases occurs a total loss of the chromosome 3 short arm [66]. In normoxic conditions, the protein encoded by the *VHL* gene serves as a recognition site for the regulatory subunits of HIF, targeting them with ubiquitin to proteasome degradation. With the loss of *VHL* in ccRCC, the degradation of HIF stops and leads to its accumulation in the cytoplasm and further migration to the nucleus where it binds to hypoxia-related genes, leading to a hypoxic response from the cell in normoxic conditions [68]. Some of the genes activated by HIF are associated with blood vessels development (Erithropoietin - EPO and Vascular endothelial growth factor - VEGF), proliferation (Platelet derived growth factor beta – PDGF-β and Transforming growth factor alpha - TGF-α), glucose metabolism (Glucose transporter 1 - GLUT 1) and metastization (Chemokine (C-X-C motif) receptor 4 - CXCR-4) (Figure 3) [69, 70].

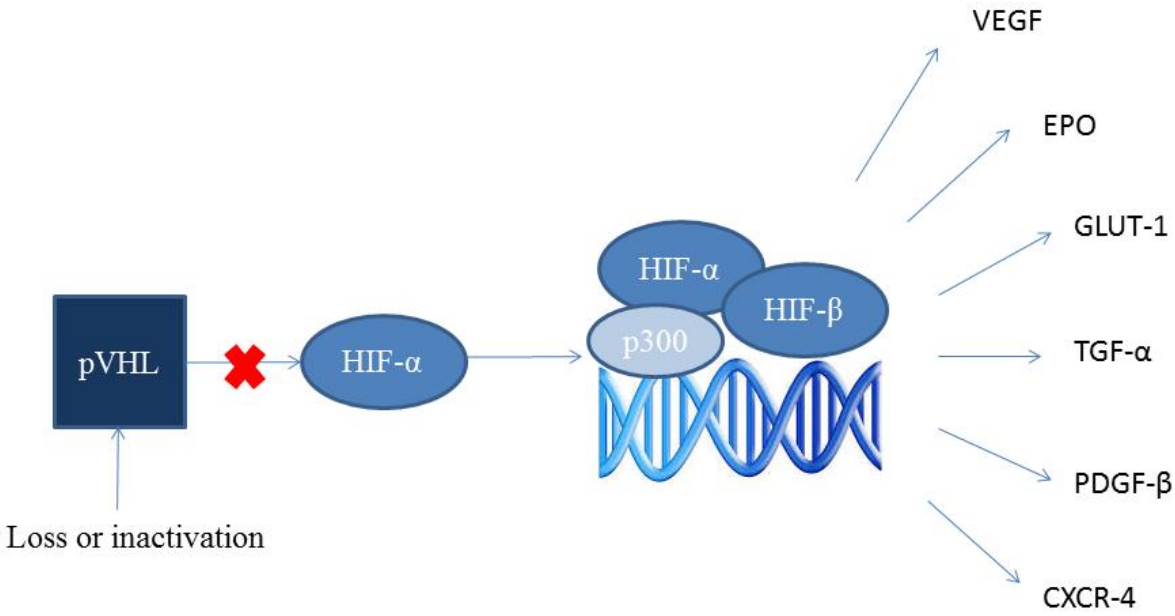

Figure 4- pVHL pathway under hypoxic conditions: the loss of VHL leads to the accumulation of HIF-α in the nucleous and consequene binding to transcription factors, which triggers a hypoxic response from the cell (Adapted from Dias, F., et al. [42]).

One of the activated genes is the transforming growth factor α (TGF-α), which is involved in the induction of cellular proliferation by activating the epidermal growth factor receptor (EGFR) [71]. The EGFR activates several signaling pathways such as MAPK/ERK and PI3K/AKT which in turn modulate genetic transcription, stimulating cellular proliferation, migration, invasion, angiogenesis and apoptosis [72-75]. EGFR belongs to the ErbB/HER family of tyrosine kinase receptors and is often dysregulated in cancer [76]. It is overexpressed in, approximately, 30% of primary tumors of several cancer types and has been associated with advanced disease, poor prognosis, limited overall survival and therapy response [77]. In RCC, multiple analysis has shown an overexpression of EGFR ranging from 40–80% [78-80]. This may occur due to the fact that pVHL is also responsible for activated EGFR downregulation by promoting efficient lysosomal degradation of the receptor, an event that during the RCC development could be compromised leading to an increase of EGFR [71]. Zhou and co-workers performed an in vitro study in ccRCC describing that EGFR half-life was approximately 1 hour in VHL-expressing ccRCC cells but approximately 3 hours in mock ccRCC cells and that both phospo-AKT and the phospo-ERK signals lasted longer in mock ccRCC cells when stimulated with epidermal growth

factor (EGF) [71]. Recently, Shen and colleagues demonstrated that EGFR is upregulated during tumor progression and specifically enriched in hypoxic tumor areas [81]. They also desmonstrated that EGFR suppresses the maturation of specific tumour-suppressor-like miRNAs in response to hypoxic stress through phosphorylation of argonaute 2 (AGO2) at Tyrosine 393 [81]. Taken together, this results demonstrate the importance of studying the VHL-EGFR pathway to further understanding of ccRCC molecular biology, and the involvement of this pathway in ccRCC tumor progression.

In recent years, the increasing knowledge of the pathways involved in ccRCC has allowed the development of new targeted therapies. The identification of alterations in *VHL* gene in ccRCC led to the development of targeted therapies such as sunitinib and sorafenib - tyrosine kinase inhibitors, as well as pazopanib (angiogenesis inhibitor) [70, 82]. Considering the facts stated before, erlotinib, an EGFR tyrosine kinase domain inhibitor, could have a benefic impact in ccRCC treatment inhibiting EGFR-related pathways since a subset of patients (~25%) do not seem to experience any clinical benefit from targeted therapies used nowadays and even patients that initially respond to therapy later develop resistance and disease progression occurs [44]. In fact, phase I and phase II clinical trials have already been performed in metastatic ccRCC patients using erlotinib alone or simultaneously with bevacizumab (angiogenesis inhibitor) but the positive impact of EGFR blockade in metastatic ccRCC patients was hard to prove [83, 84]. These “failures” of targeted therapies show us the necessity of further investigating the mechanisms involved in innate or acquired resistance and also the molecular events crucial for the progression of this type of cancer which, ultimately, would allow the development of new effective targeted therapies.

As stated before, miRNAs may be way to unveil acquired resistance mechanisms and ultimately be seen as therapeutic options [85]. In ccRCC, as a consequence of *VHL* loss and EGFR activation some miRNAs present altered expression patterns such as miRNA-21 and miRNA-210 (Figure 4) [30].

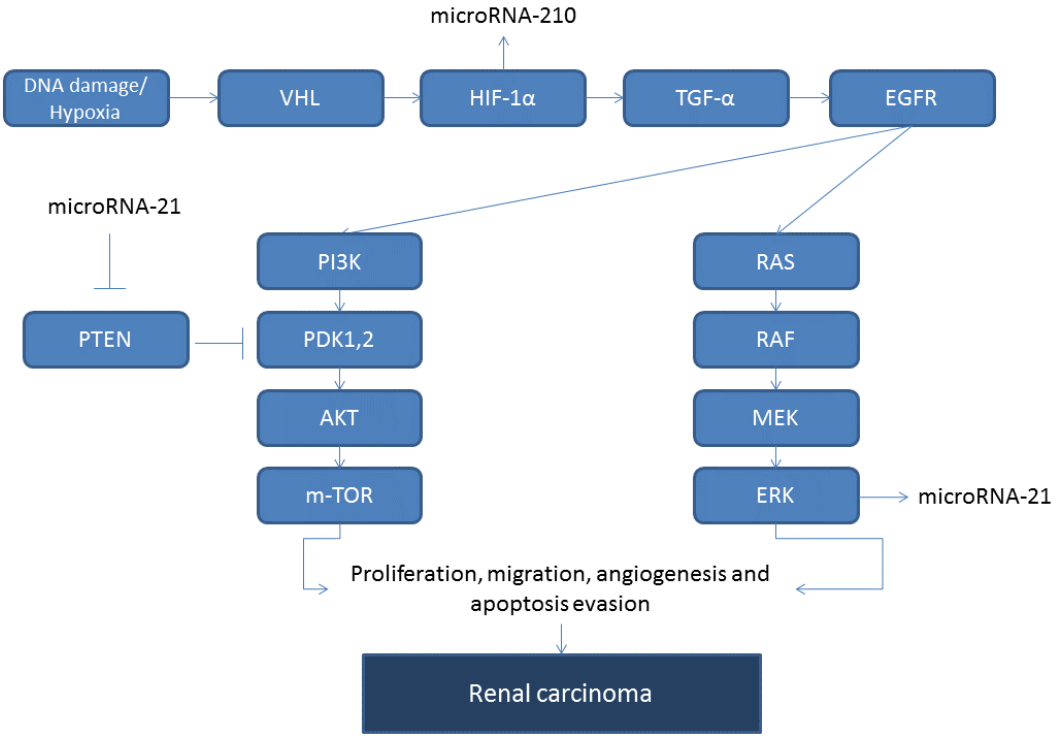

Figure 5 - Link between the VHL and EGFR pathways and microRNAs involved (Adapted from Dias, F., et al. [32]).

MiRNA-21 has been described as an oncomicroRNA with an important role in cancer development [86, 87]. In a variety of cancer cell lines, overexpression of miR-21 was able to increase cell proliferation, migration, invasion and survival, while its suppression could induce apoptosis and repress cell proliferation and invasion capacity [88]. MiRNA-21 transcription can be induced by EGFR-related AP1 and NF-kB transcription factors and MAPK/ERK signaling pathway [89, 90]. In RCC, microRNA-21 was indicated as a potential diagnosis and prognosis biomarker since it is overexpressed in tumor samples when compared with normal kidney tissue and higher miRNA-21 levels were associated with higher stage and grade of RCC tumors [91]. Since one of miRNA-21 targets is PTEN, a tumor-suppressing phosphatase that antagonizes the function of PI3K and negatively regulates AKT activity, its overexpression has been associated with ErBb family targeted therapies acquired resistance in lung, breast and gastric cancer studies [42, 92, 93]. High

levels of miRNA-21 leads to a PTEN downregulation and consequently to an overactivation of AKT signaling pathway [93].

MiRNA-210 is a miRNA that is regulated by HIF-1α [94]. HIF-1α accumulation in the cell is a key event in RCC due to *VHL* loss, therefore higher levels of this miRNA are expected in RCC patients [95]. In fact, Valera and coworkers described an overexpression of miRNA-210 in RCC patients and demonstrated that increased expression levels of miR-210 are associated with higher Fuhrman nuclear grade tumors and tumors with lymph node metastasis [95]. Some reports indicate that miRNA-210 may control c-Myc activity by downregulating MNT expression, a c-Myc antagonist [96-98]. On the one hand, c-Myc promotes cell cycle entry and when dysregulated contributes to tumor formation due to its bHLHZip domain, which mediates heterodimerization with Max and DNA binding [99]. The Myc–Max heterodimer is able to bind DNA at the E-box consensus sequence CANNTG and activate transcription [100]. On the other hand, MNT is also a Max interacting protein but with a transcriptional repressor activity, antagonizing Myc-dependent cell proliferation [101]. MNT seems to have an important role in cell cycle entry, since cells lacking MNT exhibited an accelerated G0 to S-phase transition which, ultimately, could lead to tumorigenesis [102]. Since c-Myc is a transcription factor highly activated through EGFR-related pathways, higher levels of miRNA-210 could represent a resistance mechanism to EGFR targeted therapies through MNT downregulation and loss of cell cycle entry control due to a higher Max availability to interact with c-Myc [103]. However, to the best of our knowledge, miRNA-210 has not yet been associated with acquired resistance to EGFR targeted therapies.

More than one miRNA may be involved in acquired resistance mechanisms for the same drug and in the same tumor model. With this being said, it is more likely that an acquired resistance to a targeted therapy would be mediated by a network of miRNAs rather than a single one, targeting multiple sites of different pathways. However, it is important to unveil miRNA-mediated resistance mechanisms for better understanding and prediction of treatment response, allowing a more personalized and effective treatment.

2. Objetives

2.1 Main objective

Characterization of a microRNA mediated resistance mechanism to erlotinib in renal cell carcinoma.

2.2 Specific objectives

- Evaluation of erlotinib and bevacizumab cellular toxicity in a renal cell carcinoma cell line.

- Establishment of an erlotinib-resistant renal cell carcinoma cell line.

- *In vitro* quantification of microRNA-21 and microRNA-210 intracellular and extracellular levels in a renal cell carcinoma cell line, FG-2, *versus* an erlotinib-resistant renal cell carcinoma cell line, FG-2R.

- *In vitro* quantification of mRNA MNT levels and its correlation with the microRNAs levels, previous mentioned, in a renal cell carcinoma cell line, FG-2, *versus* an erlotinib-resistant renal cell carcinoma cell line, FG-2R.

- Determination of the functional relationship between microRNA-210 and MNT.

- *In vitro* evaluation of the microRNA-210 mediated resistance mechanism.

3. Matherial and Methods

3.1 Cell line characterization

For the present study was used one cell line, FG-2, described as a metastatic RCC cell line and kindly provided by Dr. Klaas Kok from Groningen University, Netherlands (Figure 6).

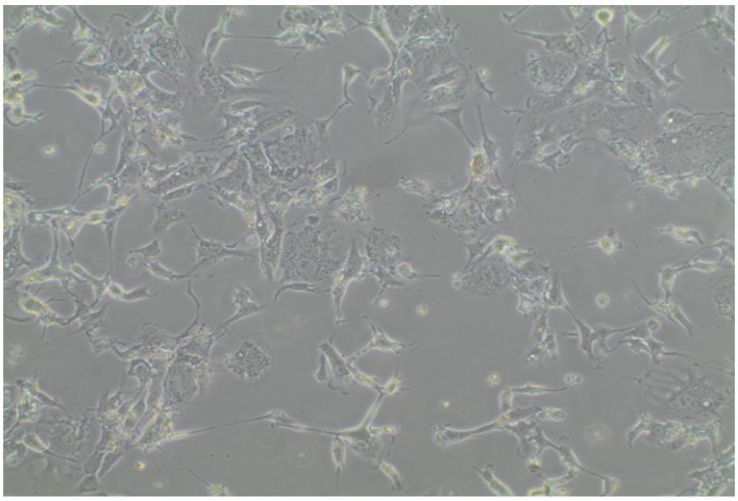

Figure 6 - Microscope image (10x) of the FG-2 cell line.

To confirm the tumoral phenotype of the cell line in study, it was performed a Fluorescence in situ Hybridization (FISH) technique to detect the absence of the *VHL* gene. It was used a specific probe for the centromere of the short arm of chromosome 3 and a specific probe for the *VHL* gene. As expected there was loss of the *VHL* gene in the FG-2 cell line (Figure 7).

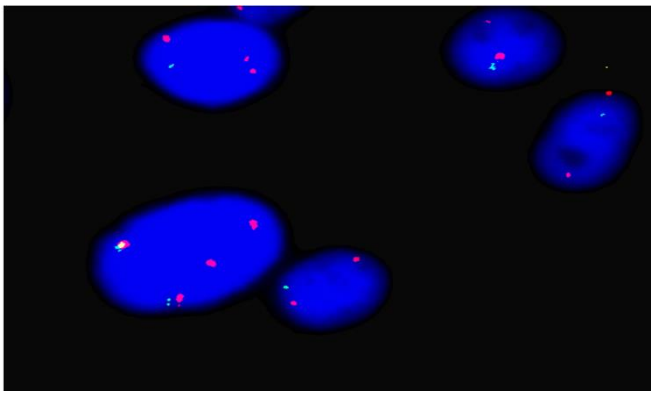

Figure 7 - Microscopic image of the chromosome 3 centromeres and VHL genes present in the FG-2 cell line. (Red dye - chromosome 3 centromere; green dye – VHL gene). Images provided by Dr. Joana Vieira from the IPO-Porto Genetic Department.

3.2 Establishment of an erlotinib-resistant renal cell carcinoma cell line and cell culture

Initially a cryopreserved vial of the FG-2 cell line was thawed. The FG-2 cell line was maintained in RPMI 1640 (1X) medium (*Gibco*®), supplemented with 10% of FBS (Fetal Bovine Serum) (*Gibco*®), and 1% of Pen-Strep (*Gibco*®). An erlotinib-resistant subline (FG-2R) was generated upon exposure of the FG-2 cell line to crescent concentrations of erlotinib. Specifically, 15 days exposure to erlotinib at 3μM, 15 days exposure to erlotinib at 5μM and 2 months exposure to erlotinib at 10μM. Cell counting using a Neubauer chamber and Tripan-Blue dye (*Gibco*®) was performed, in the presence and absence of erlotinib, at the end of the erlotinib-resistant renal cell carcinoma cell line establishment to confirm the resistance to the drug. Then, both cell lines were maintained in a 5% CO_2 incubator at 37ºC during all phases of the study.

When the desired confluence was achieved (80-90%) the medium, in which the cells were being cultured, was collected for miRNA extraction and the cells were trypsinized, using 0.05 % trypsin-EDTA (1×) (*Gibco*®) and counted using a Neubauer chamber and Tripan-Blue dye (*Gibco*®). After counting, approximately 1.5 million cells were centrifuged to form a pellet for either miRNA or mRNA extraction and the remaining cells were kept in culture.

This procedure was repeated until ten cell and medium microRNA extractions and ten cell mRNA extractions were performed for the FG-2 cell line. Taking in consideration the FG-2R cell line, this procedure was performed at the end of the 15 days exposure to erlotinib at 5μM, at the end of 1 month exposure to erlotinib at 10μM and at the end of the establishment of the erlotinib-resistant renal cell carcinoma cell line. At least three cell and medium microRNA extractions and three mRNA extractions were performed at each phase mentioned.

3.3 MicroRNA and mRNA extraction and cDNA synthesis

MicroRNA extraction from the cells and respective medium was performed using the *GRS microRNA kit* (*Grisp*®) according to manufacture instructions. After isolation, *NanoDrop*® *ND-1000* spectrophotometer was used to determine miRNA concentration and purity by measuring absorbance at 260 and 280 nm. The miRNA samples were then used as templates for cDNA synthesis using a *Taqman*®*MicroRNA Reverse Transcription kit* (*Applied Biosystems*®) and sequence-specific primers for miRNA-21, miRNA-210 and functioning as endogenous controls, for RNU-6B e RNU-48. The mRNA extraction was performed using the *GeneJET*™ *RNA purification kit* (*Thermo Scientific*®) according to manufacture instructions. After isolation, mRNA concentration and purity were measured at 260 and 280 nm using the *NanoDrop*® *ND-1000* spectrophotometer. The mRNA samples were then used as templates for cDNA synthesis using *High-Capacity RNA-to-cDNA Kit* (*Applied Biosystems*®).

3.4 Real-time PCR relative quantification

The miRNA and mRNA expression was analyzed by quantitative real-time PCR. The reactions were carried out on a StepOne™qPCR Real-Time PCR machine, containing 1X Master mix (*Applied Biosystems*®), with 1X probes (*TaqMan*® *microRNA Expression Assays,* miR-21:TM000397, miR-210:TM000512, *TaqMan*® *microRNA Control Assays,* RNU-6B: TM-001093, RNU-48: TM-001006 and *TaqMan*® *Gene Expression Assays*, MNT: Hs00232758, c-Myc: Hs00153408, B2M: Hs00187842, *Applied Biosystems*®) and a cDNA sample. RNU-6B and RNU-48 were used and quantified to determine which one had the most constant expression levels in the microRNA tested samples to normalize results and function as endogenous control. B2M was used as an endogenous control to mRNA tested samples. Data analysis was performed using *StepOne™ Sofware v2.2* (*Applied Biosystems*®).

3.5 Bevacizumab and erlotinib cellular toxicity test

In this study, 100000 cells of the FG-2 cell line were cultured in 12-well plate. After 24h, cells were administered with DMSO (erlotinib dilution solution), NaCl 0,9% (bevacizumab dilution solution), DMSO and NaCl 0,9%, erlotinib at a final concentration of 10µM, bevacizumab at a final concentration of 150 µg/mL or erlotinib at a final concentration of 10µM and bevacizumab at a final concentration of 150 µg/mL. Each condition was performed in triplicate. After 72h of administration, cells were trypsinizated and counted using, once again, a Neubauer chamber and Tripan-Blue dye (*Gibco*®). This experience was performed in duplicate.

Optimal concentrations of erlotinib and bevacizumab were determined prior to this study in similar fashion, using crescent concentrations.

3.6 Analysis of microRNA-210 and MNT functional relationship

On the present study, 150000 cells of the FG-2 cell line were cultured in 24-well plate and reversely transfected with a scrambled sequence (kindly provided by Dr. Germana Zaccagnini) or with microRNA-210 *mirVana*® miRNA inhibitor (MH10516, *Applied Biosystems*®), using ScreenFect A reagent (*InCella*®) according to manufacture instructions. After 24h, cells were trypsinizated and microRNA and mRNA extraction was performed for each condition for further analysis by real-time PCR of microRNA-210, RNU-6B, MNT and B2M levels. This experience was performed in triplicate.

3.7 FG-2 cell line conditioning

Two different experiments using FG-2R conditioned medium were performed in the FG-2 cell line. First, 550000 cells of the FG-2 cell line were cultured in 6-well plate. After 24h, growth medium was removed and either 5mL of FG-2R conditioned medium or, as an experiment control, 5mL of FG-2 conditioned medium was added. Both conditions were performed in duplicate. After 8h, cells were trypsinizated and microRNA extraction was performed for further analysis of microRNA-21, microRNA-210 and RNU-6B levels by real-time PCR, as previously mentioned. Secondly, an experiment was performed to test if the FG-2 cell line conditioning was time-dependent. For that matter, 500000 cells of the FG-2 cell line were cultured in 6-well plate with FG-2R conditioned medium in a ratio of 1:1 with FG-2 growth medium during 2 days, 1 week or 2 weeks. Each condition was performed in duplicate. Cells microRNA extraction and quantification of microRNA-21, microRNA-210 and RNU-6B levels by real-time PCR were performed at each exposure time of the study.

3.8 Erlotinib response of the FG-2R cell line transfected with a microRNA-210 inhibitor

For this study, 70000 cells of the FG-2R cell line were cultured in 24-well plate with growth medium supplemented with erlotinib at a final concentration of 10µM and reversely transfected with a scrambled sequence (kindly provided by Dr. Germana Zaccagnini) or with microRNA-210 mirVana® miRNA inhibitor (MH10516, *Applied Biosystems*®), using ScreenFect A reagent (*InCella*®) according to manufacturer recommendations. After 72h, erlotinib response was evaluated by viable cell counting using a Neubauer chamber and Tripan-Blue dye (*Gibco*®) after trypsinization. This experiment was performed in triplicate.

3.9 Co-Immunoprecipitation

Co-immunoprecipitation was performed to analyze protein-protein interaction between c-Myc and Max in G1 phase of the cell cycle in FG-2, FG-2R and FG-2R transfected with microRNA-210 *mirVana*® miRNA inhibitor (MH10516, *Applied Biosystems*®). First, 750000 cells of the FG-2R cell line were cultured in 6-well plate and reversely transfected with microRNA-210 *mirVana*® miRNA inhibitor (MH10516, *Applied Biosystems*®), using ScreenFect A reagent (*InCella*®) according to manufacturer recommendations, for c-Myc and Max interaction analysis after 72h. Simultaneously, FG-2 and FG-2R cell lines were grown to confluence in a T25. After 24h of FG-2R transfection, in all conditions growth medium was switched from medium containing 10% FBS to medium containing 0.1% FBS. Cells were maintained in 0.1% FBS for 2–3 days before stimulating with medium containing 10% FBS for 6h, which allowed G1 phase entry. After this period of time, cells were washed with PBS (*Invitrogen*™) and cell lysis was performed using a mix of proteases inhibitor and co-immunoprecipitation buffer in a ratio of 1:200. Protein extracts were stored at -20ºC. After protein quantification by bicinchoninic acid method (BCA™ Protein Assay, *Pierce*), 30 μg of total protein from each condition was stored at -20ºC for western blot analysis and 150 μg of total protein of each condition was incubated *overnight* at 4ºC with 4 μg of anti-Max (C-17, *Santa Cruz Biotechnology*®) or with 4 μg of mouse IgGs, acting as control. After *overnight* incubation, samples were centrifugated 10 minutes at 13000 rpm. Subsequently, 10 μL of magnetic beads were added to each condition and incubated during 1h while in rotation. After this step, magnetic beads were washed five times with co-immunoprecipitation buffer and then GLB (1X) and DTT, at a final concentration of 0,1M, was added to the beads for protein elution. Simultaneously, these two reagents were also added to the 30 μg of total protein stored at -20ºC for western blot analysis from each condition (FG-2, FG-2R and FG-2R transfected with microRNA-210 inhibitor). Samples from each condition were heated for 10 minutes at 50ºC and then magnetic beads were removed from solution. Afterwards, samples were loaded into a 14% SDS-PAGE polyacrylamide gel and running conditions were 200 V and 30 mAp. Once the dye front ran off the bottom of the gel, the gel was removed and proteins were transferred to a nitrocellulose membrane using Trans-Blot® Turbo™ Kit (*BIO-RAD*®) and Trans-Blot® Turbo™ Transfer System (*BIO-RAD*®), according to manufacturer recommendations. After the transfer was completed, a ponceau solution (1X) was added to the nitrocellulose

membrane for co-immunoprecipitation and transfer efficiency control. Subsequently, the nitrocellulose membrane was divided into two according to the molecular weight of the proteins in study and ponceau solution was removed. Then, the membranes were incubated and blocked during 1 hour in 5% skim milk in TBS-TritonX 0,2%. After this step, membranes were incubated *overnight* with anti-c-Myc primary antibody (9E10, *Santa Cruz Biotechnology*®) on a 1:100 dilutions in 2% skim milk in TBS-TritonX 0,2% or with anti-Max primary antibody (C-17, *Santa Cruz Biotechnology*®) on a 1:200 dilutions in 2% skim milk in TBS-TritonX 0,2%. On the next day, membranes were washed with TBS-TritonX 0,2% and incubated during 1 hour with the respective secondary antibody on a 1:10 000 dilutions in 2% skim milk in TBS-TritonX 0,2%. Finally, membranes were washed in TBS-TritonX 0,2% and incubated with Clarity™ Western ECL Substrate (*BIO-RAD*®), according to manufacturer recommendations, for visualization on a ChemiDoc machine using ImageLab (*BIO-RAD*®).

3.10 Statistical Analysis

Statistical analysis was performed using IBM®SPSS®Statistics for Windows (Version 20.0). Livak method ($2^{-\Delta\Delta Ct}$) and t´ student test was used to evaluate the differences in the expression levels of the normalized miRNAs and mRNAs. Western blot analysis was performed using ImageLab software (*BIO-RAD*®).

4. Results

4.1 Bevacizumab and erlotinib cellular toxicity test

In vitro studies were performed in the FG-2 cell line to determine erlotinib and bevacizumab cellular toxicity, separately and simultaneously. In figures 8 and 9, are represented the number of viable and non-viable cells obtained in response to different concentrations of bevacizumab and erlotinib. Moreover, in figure 10 we can observe the effect of bevacizumab and erlotinib, when used simultaneously, in cell viability. Regarding the results acquired for bevacizumab exposure of the FG-2 cell line, no differences were observed in cell viability when comparing the condition control, where cells were exposed to NaCl 0,9%, and any of other conditions where the FG-2 cell line was exposed to different bevacizumab concentrations (Figure 8).

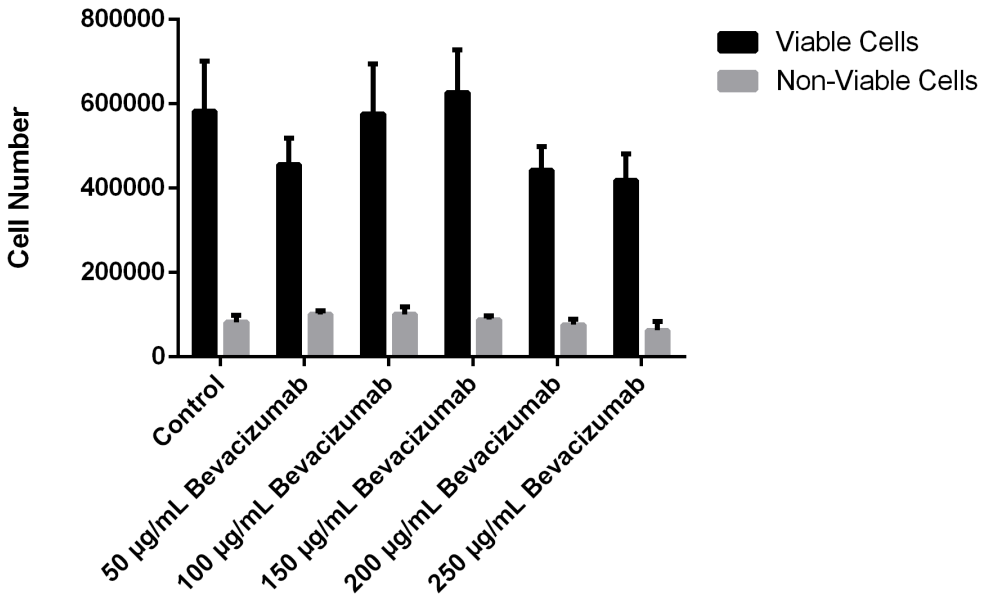

Figure 8 – Number of viable and non-viable FG-2 cells after 72h exposure to different bevacizumab concentrations (Mean ± SEM).

According to the results obtained for erlotinib exposure of the FG-2 cell line, there were statistical significant differences in cell viability between the control condition, where cells were exposed to DMSO, and the 10µM erlotinib exposure condition (*P*= 0.006) (Figure 9). Since this condition led to, approximately, the death of 50% of the FG-2 cells we considered as our comparison condition.

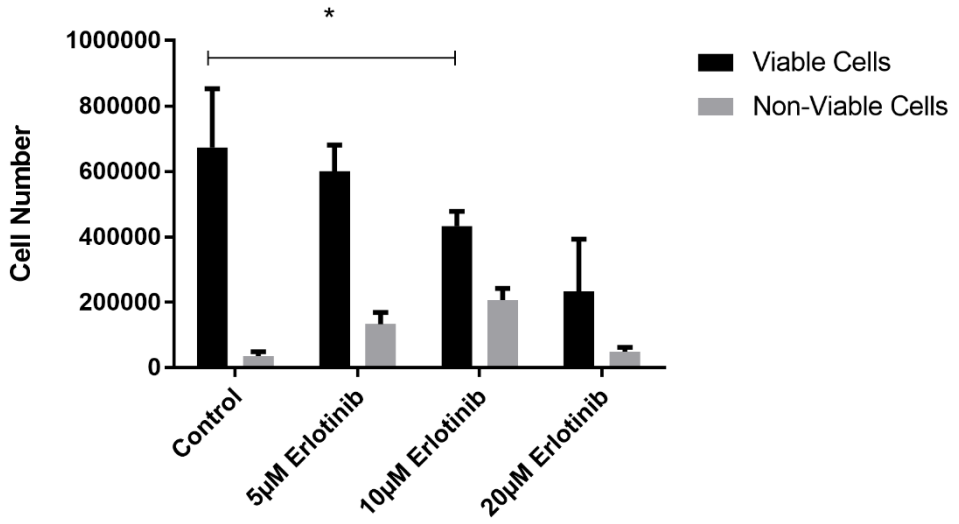

Figure 9 - Number of viable and non-viable FG-2 cells after 72h exposure to different erlotinib concentrations (Mean ± SEM; *P≤0.050).

Additionally, the FG-2 cell line was also exposed to bevacizumab and erlotinib simultaneously. Statistical significant differences in cell viability were found between the 10µM erlotinib exposure condition and the 10µM erlotinib + 150µg/mL bevacizumab exposure condition (*P*= 0.007), revealing a synergic effect of these two drugs (Figure 10).

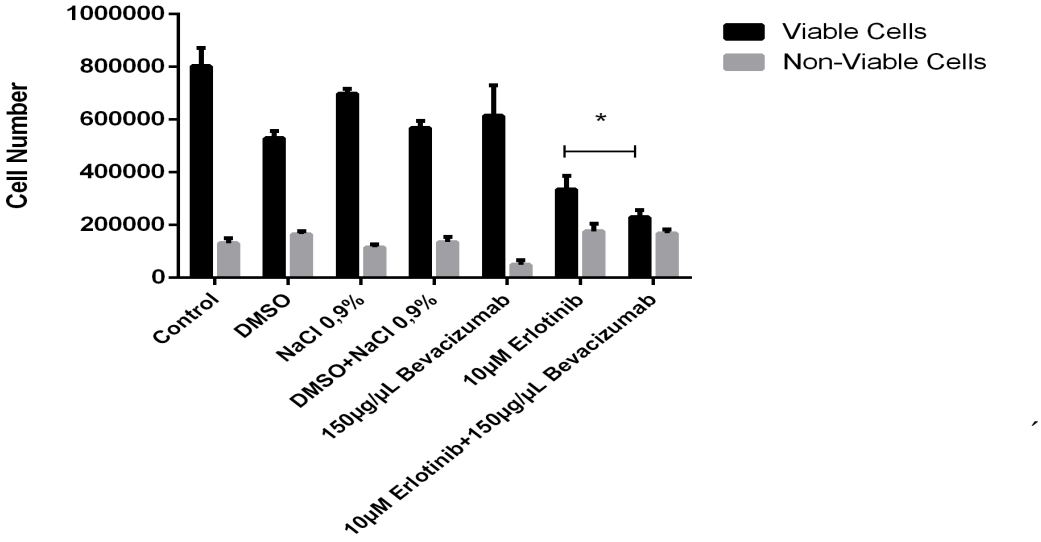

Figure 10 - Number of viable and non-viable FG-2 cells after 72h exposure to bevacizumab and erlotinib, separately or simultaneously (Mean ± SEM; *P≤0.050).

4.2 MicroRNA and mRNA levels analysis between FG-2 and FG-2R cell lines

Since the miRNA levels were going to be analyzed using the comparative CT method, two miRNAs, RNU6B and RNU48, were quantified to determine which would be an adequate endogenous control in our study models. We observed that RNU6B presented less variation between the FG-2 and FG-2R cell samples than RNU48 (SEM (RNU6B) = 1.09 vs SEM (RNU48) = 1.93) and also between our FG-2 and FG-2R medium samples (SEM (RNU6B) = 1.18 vs SEM (RNU48) = 1.37) (Figure 11). MRNA levels were also quantified using the comparative CT method and B2M was used as our endogenous control since previous results demonstrated that its expression was stable and constant among samples.

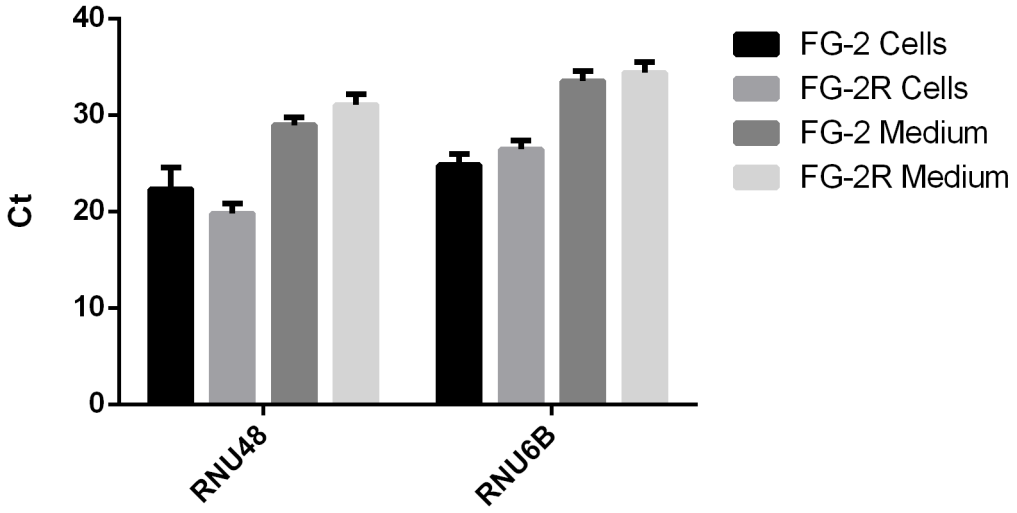

Figure 11 - Comparison between intracellular and extracellular expression levels of RNU-6B and RNU-48 in FG-2 and FG-2R cell lines and respective medium (Mean ± SEM).

In figures 12 to 14, are represented the results relative to the intracellular and extracellular expression levels of miRNA-21 and miRNA-210 and intracellular levels of MNT and c-Myc in FG-2 and FG-2R cell lines.

According to the results regarding miRNA-21, we observed a trend for higher intracellular levels of this miRNA in the FG-2R cell line at end of the second month of exposure to 10µM of erlotinib when compared with the FG-2 cell line (P= 0.185). However, we did not found statistical significant differences between the extracellular levels (P= 0.319) (Figure 12).

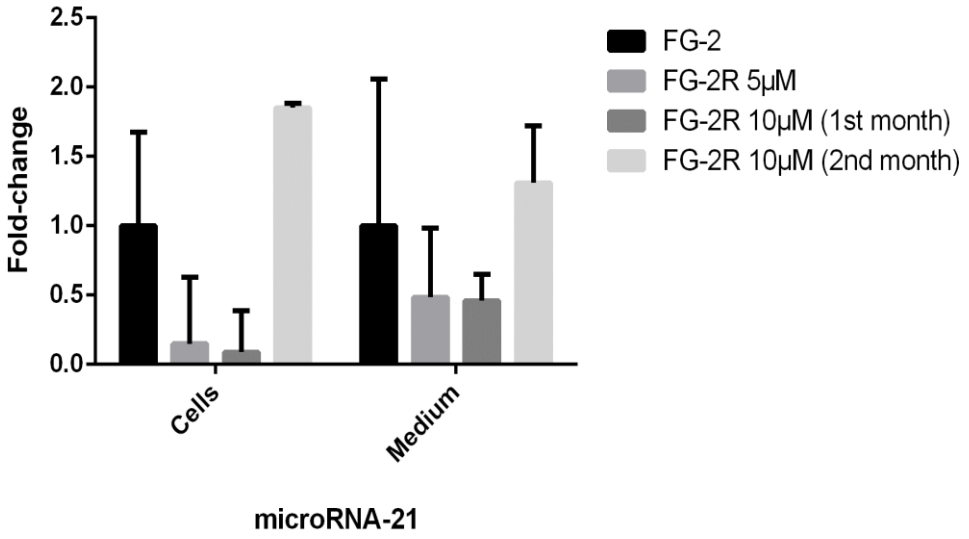

Figure 12 – miRNA-21 intracellular and extracellular levels fold change between FG-2 cell line and FG-2R cell line at three different stages of establishment (Mean ± SEM).

Regarding miRNA-210, at the end of the FG-2R cell line establishment (2nd month of exposure to 10µM erlotinib) we observed not only a 6.26 fold-increase in miRNA-210 intracellular levels (*P*= 0.009) but also a 19 fold-increase in miRNA-210 extracellular levels (*P*= 0.017) when compared with the FG-2 cell line (Figure 13).

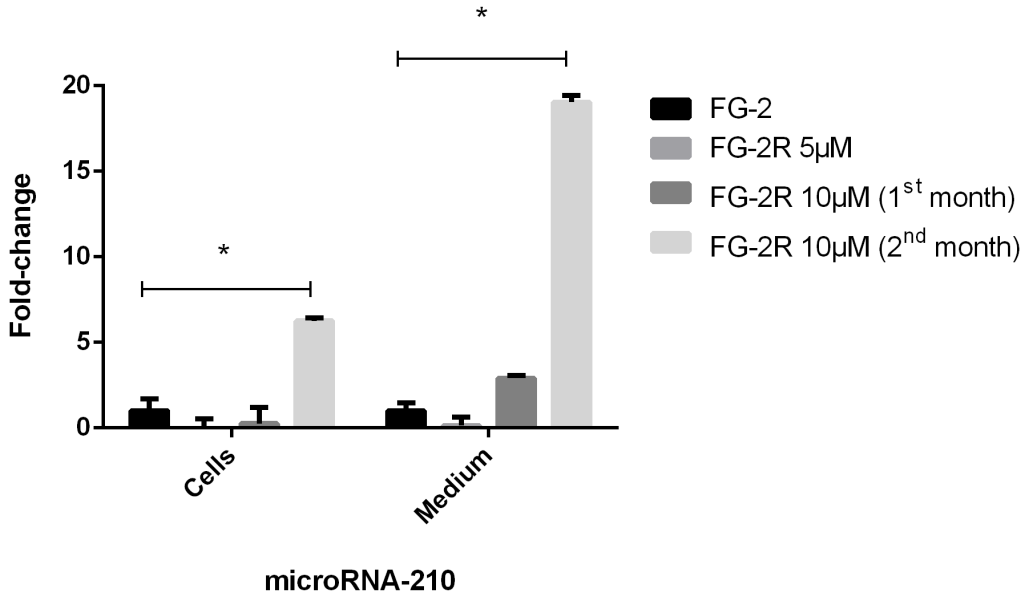

Figure 13 - miRNA-210 intracellular and extracellular levels fold change between FG-2 cell line and FG-2R cell line at three different stages of establishment (Mean ± SEM, *P≤0.050).

According to the results obtained for mRNA MNT and c-Myc levels quantification, we observed a statistical significant decrease in the intracellular levels of both transcripts at the endpoint of the FG-2R cell line establishment when compared with the FG-2 cell line ($P< 0.001$ in both transcripts) (Figure 14).

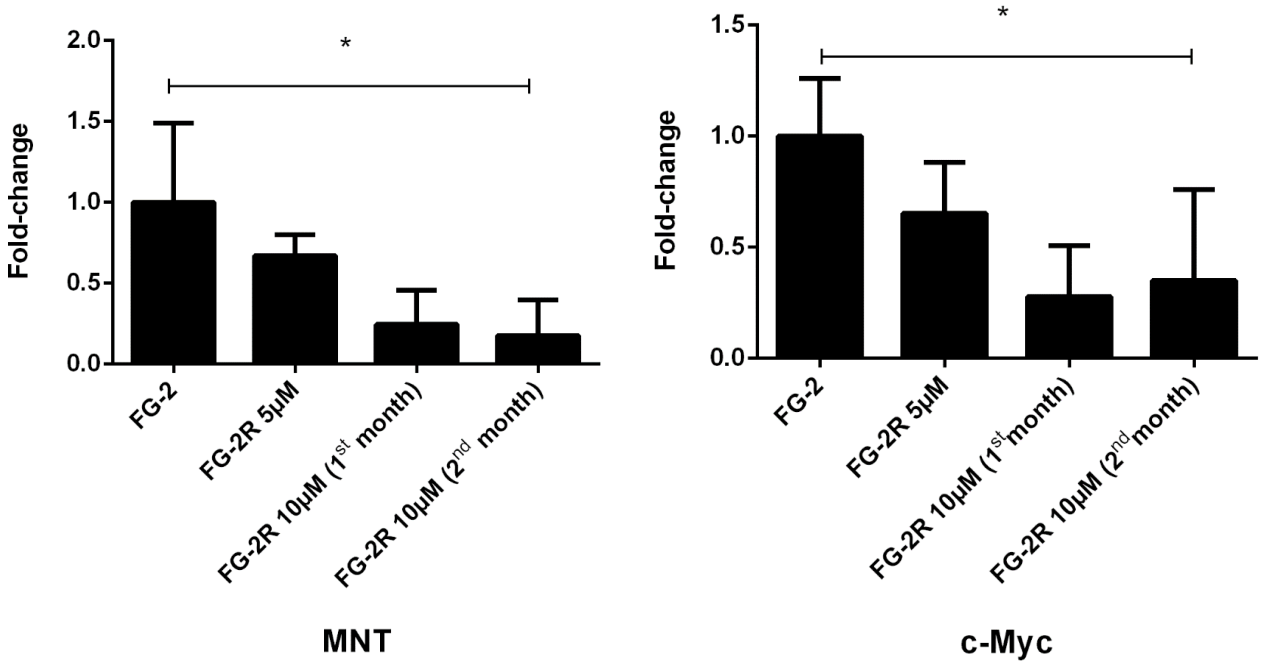

Figure 14 – C-Myc and MNT intracellular levels fold change between FG-2 cell line and FG-2R cell line at three different stages of establishment (Mean ± SEM, *P≤0.050).

4.3 MicroRNA-210-dependent erlotinib resistance mechanism

Since miRNA-210 intracellular and extracellular levels were upregulated in the FG-2R cell line when compared with the FG-2 cell line, miRNA-210 knockdown experiments were performed in the FG-2R cell line to verify its influence in erlotinib resistance.

According to the results obtained of the FG-2R cell line response to erlotinib after miRNA-210 inhibitor transfection, we observed a statistical significant decrease in the cell viability of the FG-2R cell line after miRNA-210 inhibitor transfection when compared with the FG-2R cell line transfected with a scramble sequence ($P= 0.009$) (Figure 15).

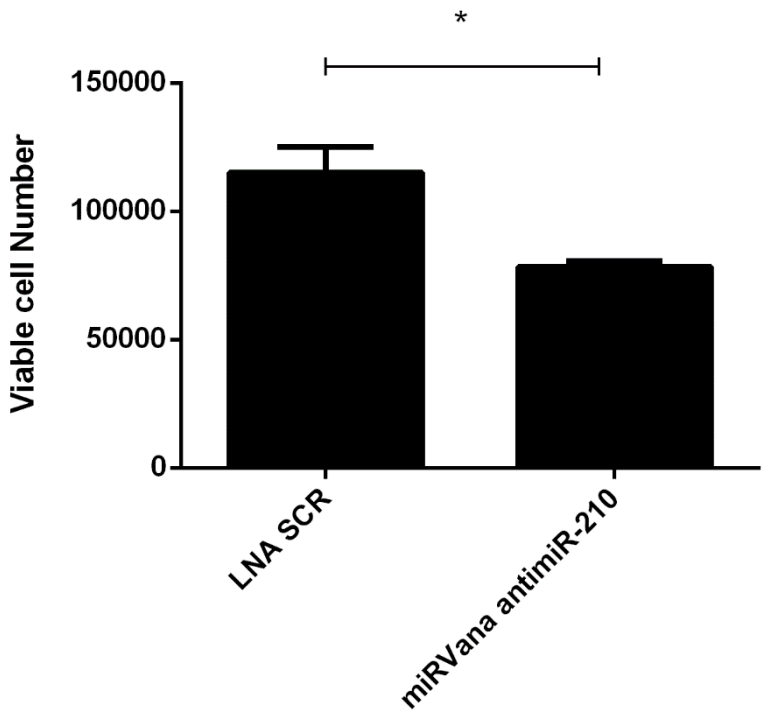

Figure 15 – Comparison between the number of viable FG-2R cells transfected with a scrambled sequence or with a miRNA-210 inhibitor after 72h exposure to erlotinib (Mean ± SEM; *P≤0.050).

To verify if FG-2R erlotinib resistance mechanism was dependent on miRNA-210 and MNT functional relationship and its possible effect on c-Myc-Max interaction in the cell cycle, different studies were performed. First, miRNA-210 and MNT functional relationship was evaluated through FG-2 cell line transfection with a miRNA-210 inhibitor. A statistical significant increase in MNT levels was observed in the FG-2 cell line transfected with a miRNA-210 inhibitor when compared with a control condition where the FG-2 cell line was transfected with a scramble sequence (P= 0.005) (Figure 16).

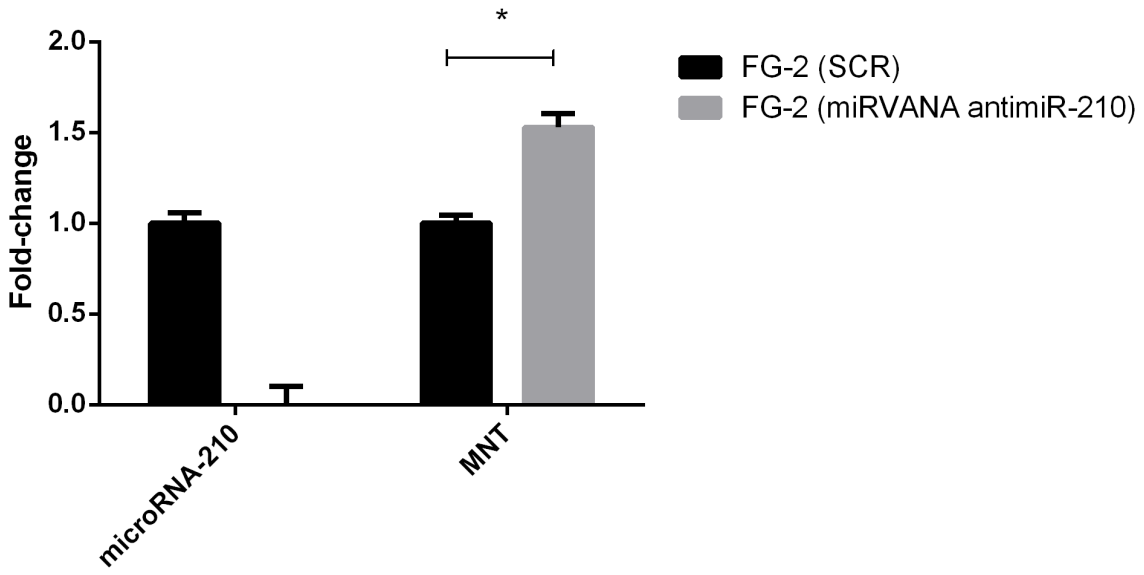

Figure 16 – MiRNA-210 and MNT intracellular levels fold change between FG-2 cell line transfected with a scramble sequence or with a miRNA-210 inhibitor (Mean ± SEM, *P≤0.050).

Secondly, c-Myc-Max interaction in G1 phase of the cell cycle was evaluated on FG-2, FG-2R and FG-2 transfected with a miRNA-210 inhibitor. According to the results, we observed an increase in c-Myc-Max interaction in FG-2R cell line when compared with FG-2 cell line (1.76 ± 0.34) but not a significant difference in c-Myc-Max interaction when comparing FG-2 transfected with a miRNA-210 inhibitor and FG-2 cell line (1.08 ± 0.76) (Figure 17).

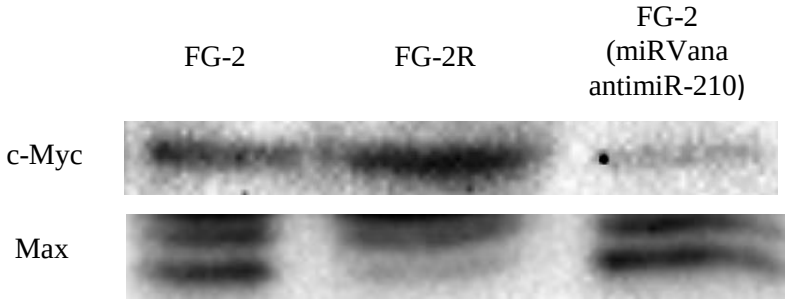

Figure 17 – Evaluation of c-Myc-Max interaction in G1 phase of the cell cycle in FG-2, FG-2R and FG-2 transfected with a miRNA-210 inhibitor.

4.4 FG-2 cell line conditioning

Experiments on the FG-2 cell line were also performed using FG-2R cell line conditioned medium to verify if any microRNA levels changes and phenotypical modulation occurred. According to the results, after 8h of exposure to FG-2R cell line conditioned medium no statistical significant differences were obtained in miRNA-21 and miRNA-210 intracellular levels when comparing FG-2 cell line exposed to FG-2 conditioned medium or FG-2R conditioned medium (Figure 18).

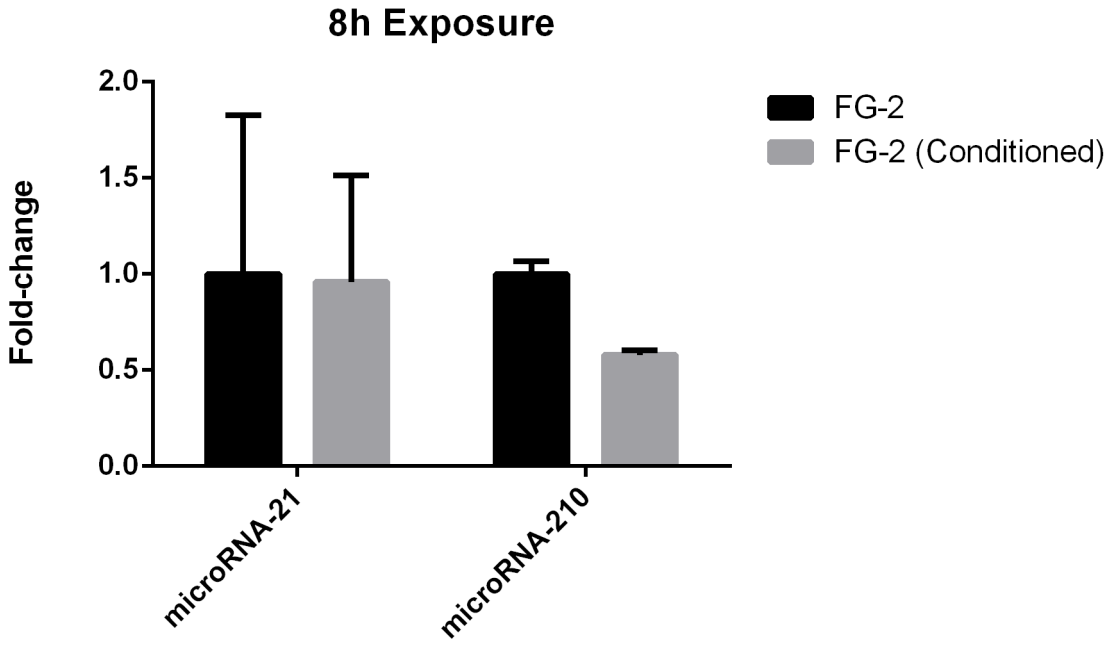

Figure 18 - MiRNA-21 and miRNA-210 intracellular levels fold change between FG-2 cell line exposed, during 8h, to FG-2 conditioned medium or FG-2R conditioned medium (Mean ± SEM).

According to the results of a longer exposure time of the FG-2 cell line to FG-2R cell line conditioned medium, also no statistical significant differences were obtained in miRNA-21 and miRNA-210 intracellular levels when comparing FG-2 and FG-2 exposed to FG-2R conditioned medium during different periods of time (Figure 19).

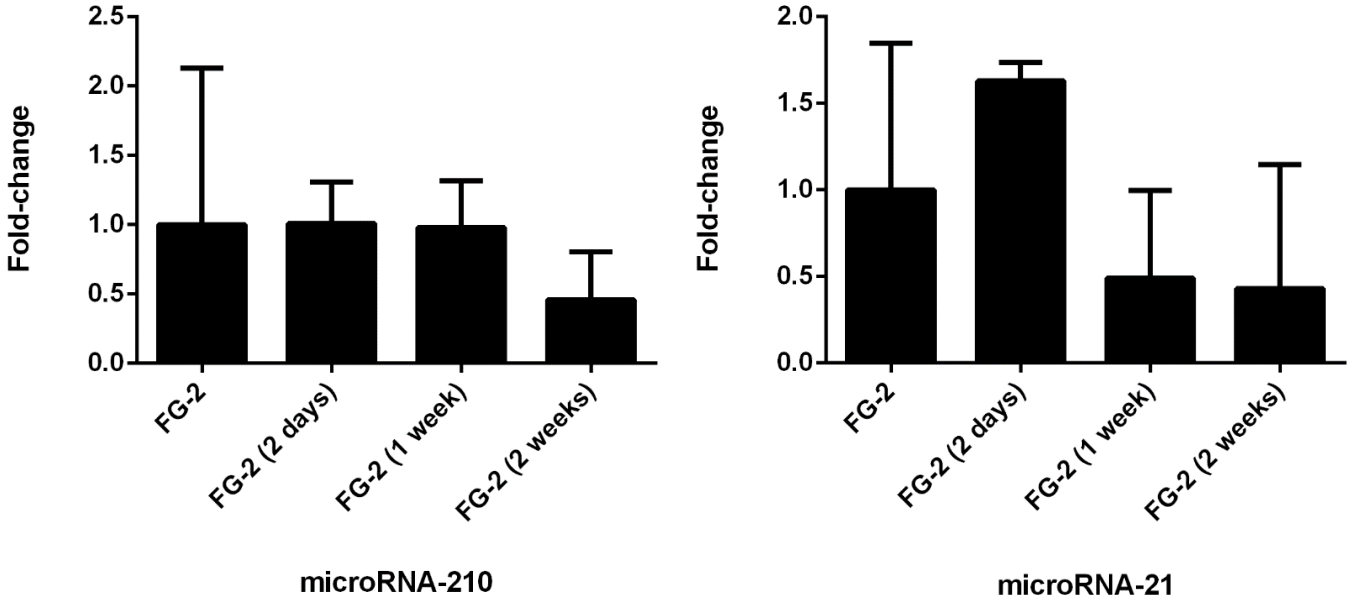

Figure 19 - MiRNA-21 and miRNA-210 intracellular levels fold change between FG-2 cell line and FG-2 cell line exposed to FG-2R conditioned medium during 2 days, 1 week or 2 weeks (Mean ± SEM).

5. Discussion

ccRCC is a neoplasia that presents an aggressive phenotype and high potential to metastasize due to its intense vascularity and angiogenic factors upregulation [53, 70]. Once the main characteristics and dependencies of this tumor type started to be elucidated, an effort was made on the development of new targeted therapies. Bevacizumab, a monoclonal antibody that inhibits VEGF, was an important step towards a more specific treatment in mRCC patients due to the constant state of hypoxia and VEGF upregulation that exists in this tumor model [68-70, 104]. The VEGF sequester performed by bevacizumab, *in vivo*, leads not only to the inhibition of VEGF-VEGFR binding on endothelial cells, reducing tumor vessel formation but also could inhibit VEGF-VEGFR binding in tumor cells, reducing the autocrine VEGF/VEGFR signaling loop and consequent activation of MAPK/ERK and PI3K/AKT signaling pathways [105, 106]. According to our *in vitro* results, bevacizumab did not have an effect on cell viability of a ccRCC cell line. However, since the positive effect of bevacizumab in mRCC patients treatment has been proven, this result may indicate that its effect on ccRCC development and progression is mainly through tumor vessel formation inhibition, an endpoint that could not be analyzed through an *in vitro* study using a viability test [104]. Additionally, this data suggests that the autocrine VEGF/VEGFR signaling loop and consequent signaling pathways activation could not be relevant to cell survival and proliferation in ccRCC or are easily surpassed by signaling pathways activation through other receptors and mechanisms. *In vitro* bevacizumab treatment overcome in ccRCC cells may be consequence of EGFR activation and consequently MAPK/ERK and PI3K/AKT related signaling pathways activation [75]. EGFR overexpression and overactivation of downstream signaling cascades have been reported and confirmed at our group in ccRCC, revealing its importance [107-110]. Since erlotinib led to a decrease in FG-2 cells viability, this indicates the key role of EGFR and related signaling pathways have on cell survival and proliferation in ccRCC. Combination therapy has presented itself as crucial approach on treating cancer because of its potential to reduce resistance to targeted therapies and improve efficacy through the inhibition of multiple receptors [111]. The combination of erlotinib and bevacizumab may be beneficial on treating RCC since they target different receptors, namely EGFR and VEGFR, that share both parallel and reciprocal downstream signaling mechanisms, such as PI3K/AKT and MAPK/ERK [105]. In fact, our *in vitro* results showed the positive effect that the

combination of erlotinib and bevacizumab may have on treating ccRCC patients since cells treated with this combined approach showed lower proliferation rates. EGFR and VEGFR crosstalk can be associated with TGF-α, which is highly expressed in ccRCC due to HIF accumulation [69, 70]. This ligand has the ability of activating EGFR and, consequently, increase the production of VEGF in human cancer cells [69, 70, 105]. Associated with this mechanism are EGFR-related MAPK/ERK and PI3K/AKT signaling cascades activation and consequent migration of three different transcription factors, STAT3, Sp1 and HIF to the VEGF promoter region [112]. Additionally, it has been suggested that inhibition of EGFR-related signaling pathways by gefitinib leads to VEGF downregulation [113, 114]. Recent work has also demonstrated that inhibition of the downstream EGFR-mediated effector mTOR reduces VEGF expression and capillary tube formation by endothelial cells [115]. Interestingly, a study associated EGFR targeted therapy resistance with increased levels of VEGF which were associated with an increase in angiogenic potential *in vitro* and tumor angiogenesis *in vivo* [112]. All these findings highlight the EGFR and VEGFR crosstalk and the common downstream signaling pathways that can influence tumor development and progression revealing the importance of using them as therapeutic targets. Unfortunately, the use of erlotinib alone or simultaneously with bevacizumab did not exhibit a consistent effect on mRCC patient´s treatment in phase I/II clinical trials [83, 84]. One of the reasons behind these results may be associated with erlotinib toxicity. Since erlotinib doses exceeding 150mg/day produced severe side effects in mRCC patients, the necessity of adjusting erlotinib dosage may lead to a greater tumor cell survival and the ability of these cells to adapt and develop resistance mechanisms [83]. Further clinical trials should also be performed using erlotinib and bevacizumab in earlier stages of RCC to verify if the synergy of these two drugs is greater and if, ultimately, a better response to treatment occurs.

MiRNAs may be a way to unveil targeted therapies resistance, allowing a better understanding of treatment response and also to perspective new therapeutic approaches. Different studies in different tumor models reported the overexpression and consequent involvement of miRNA-21 in EGFR targeted therapies resistance [42, 92, 93]. Since miRNA-21 targets PTEN, which negatively regulates AKT activity, its overexpression would lead to PTEN downregulation and consequent downstream activation of PI3K/AKT signaling pathway, surpassing the inhibitory effect of EGFR targeted therapies and leading to cell survival and proliferation [42]. According to our results, miRNA-21 does not seem to be involved in any resistance mechanism in an erlotinib-resistant RCC cell line. However,

on the other hand, miRNA-210 seems to be relevant to the erlotinib-resistant phenotype acquisitionsince it was overexpressed in an erlotinib-resistant RCC cell line, FG-2R, when compared with the parental RCC cell line, FG-2. Upon miRNA-210 knockdown on the erlotinib-resistant RCC cell line, sensitivity to erlotinib was restored confirming the importance of this miRNA on erlotinib resistance development. This result could be important to understand why erlotinib alone or in combination with bevacizumab did not have any benefic effect on mRCC patient's treatment [83, 84]. An overexpression of miRNA-210 was reported in RCC patients and higher levels of this miRNA were associated with higher tumor grades and metastasis [95]. Zhao and coworkers also showed that miRNA-210 was overexpressed in serum samples of patients with RCC compared with healthy controls and at our group miRNA-210 plasma levels were also associated with greater tumor size and metastasis [116]. Taken together, high levels of miRNA-210 could be associated with the non-response to erlotinib by mRCC patients. Since miRNAs are present in most biologic fluids and are highly stable in circulation, mainly due to being released inside of exosomes, it is important to consider them as one of the top candidates for circulating biomarkers [33-35]. In fact, miRNA-210 could represent a good biomarker for erlotinib treatment response, after *in vivo* studies confirmed and validated our results, allowing a more precise and personalized treatment. Ultimately, a miRNA-210 inhibitor could be used in the future as adjuvant therapy to erlotinib when treating RCC patients since recent *in vivo* studies have been demonstrating the ability of miRNAs to influence cancer growth and progression. For example, Ohno and co-workers efficiently deliver, *in vivo*, let-7a, a miRNA that functions as a tumor suppressor, to breast cancer cells in $RAG2^{-/-}$ mice by loading it to modified exosomes [117, 118]. The modified exosomes had GE11 peptide, an EGFR agonist, in their membranes to specifically deliver exosomal content to EGFR-expressing breast cancer cells [117]. This treatment suppressed tumor growth and no major organ damage was detected in the injected mice.

Our *in vitro* study also showed that combined with the increase of miRNA-210 levels, MNT mRNA (a miRNA-210 target) levels were downregulated in the erlotinib-resistant RCC cell line when compared with the parental RCC cell line [96]. The functional relationship of miRNA-210 and MNT was confirmed upon miRNA-210 knockdown in the RCC cell line, FG-2. MNT is a known antagonist of c-Myc, with a transcriptional repressor activity [101]. MNT and c-Myc compete for Max binding, an essential partner, mainly during the G1/S phase of the cell cycle inhibiting or promoting cell cycle entry and

progression, respectively [119]. On the one hand, c-Myc-Max heterodimers bind DNA and activate transcription of genes such as cyclins D1 and D2, cyclin E, CDK4 (cyclin-dependent kinase 4) and cyclin B1 [119, 120]. On the other hand, MNT-Max heterodimers also have the ability of DNA binding but with a transcriptional repressor activity [101]. Concomitantly with the upregulation of miRNA-210 levels and downregulation of MNT mRNA levels in the erlotinib-resistant RCC cell line, we verified an increase in c-Myc-Max interaction in the G1 phase of the cell cycle when compared with the parental RCC cell line. A greater c-Myc-Max interaction surpasses, at some degree, erlotinib EGFR inhibition, since c-Myc is a transcription factor highly activated through EGFR-related pathways [103]. This complex has the ability to regulate the transcription of many genes associated with different cellular processes, and, when dysregulated leads to a loss of cell cycle control and proliferation, metabolism dysregulation and metastatic potential increase [121]. Unfortunately, upon miRNA-210 knockdown we could not obtained a consistent result regarding the expected decrease of c-Myc-Max interaction.

Another interesting result was the presence of only the p22 Max isoform in the erlotinib-resistant RCC cell line when compared with the presence of both p21 and p22 Max isoforms in the parental RCC cell line. Although not many studies have been developed on this subject and the specific function of these two Max isoforms is yet unclear, reports showed that p22 Max isoform can form homodimers and silently bind DNA while p21 Max isoform can not [122]. Additionally, c-Myc-p22 Max heterodimer binds DNA slightly better than c-Myc–p21 Max heterodimer [122]. Taking in consideration both facts, we verify that in the erlotinib-resistant RCC cell line we, not only have a greater c-Myc-Max interaction, but also this interaction is mainly between c-Myc-p22 Max isoform which has a superior ability of DNA binding and, ultimately could lead to a greater effect on gene expression and cell processes dysregulation. Moreover, the ability of p22 Max isoform to form homodimers and silently bind DNA could allow the erlotinib-resistant RCC cell line to regulate, at some level, all the dysregulated events generated from a greater c-Myc-Max interaction [122].

Regarding the conditioning of the RCC cell line, FG-2, with the erlotinib-resistant RCC cell line conditioned medium did not exhibit significant results. No intracellular miRNA-21 and miRNA-210 levels changes were obtained in the FG-2 cell line in any of the exposure times to FG-2R conditioned medium and also no phenotypic alterations were observed. MiRNAs have been described as important mediators of intercellular communication since they are highly stable when in circulation and can modulate recipient cells, systemically [92,

123]. Their high stability and protection against ribonucleases is mainly due to the fact that miRNAs are packaged in lipid vesicles, exosomes, or associated with proteins, AGO2 mostly, or even with lipoprotein complexes [123]. Recently, many studies have been focusing on cancer exosomes-mediated cell communication and the ability of these nanovesicles to transport miRNAs in their lumen, allowing them to interfere with different cellular processes in the recipient cells [37, 123, 124]. Regarding our results, exosomes containing microRNAs present in the conditioned medium possibly are not being internalized by the FG-2 cell line and no microRNAs levels changes occurs. Another possible justification is that although is fascinating the idea of miRNAs having an important role on tumor progression in a local and systemic manner, mainly due to being shuttled inside of exosomes, recent reports showed that miRNAs in circulation are mainly associated with AGO2 and, to a lesser extent, with other AGO proteins [125, 126]. However, how miRNAs-AGO2 complexes are exported from the cell is not fully clarified but it has been proposed that these complexes are released by death or apoptotic cells and remain in the extracellular space because of the high stability of the AGO2 protein [123]. Associating these findings with our FG-2 cell line conditioning results, this may indicate that even with higher extracellular levels of miRNA-210 in the FG-2R cell line when compared with the FG-2 cell line, we could not obtain miRNA levels changes due to miRNAs being mainly associated with AGO2 and having no ability of modulating recipient cells on this manner.

In conclusion, miRNA-210 seems to be involved in an erlotinib resistance mechanism in RCC by influencing c-Myc-Max interaction and, ultimately, leading to cell cycle entry and cell proliferation.

6. Conclusion and Future Perspectives

RCC represents the most lethal urologic neoplasia mainly due to the non-existence of a standard screening test for the early detection and also due to the lack of therapeutic options with a beneficial effect in advanced RCC treatment during a significant period of time.

The use of targeted therapies to key molecules involved in RCC development has been revolutionizing cancer treatment. The combination of these therapies has allowed to surpass treatment limitations such as treatment resistance and improves efficacy through the inhibition of different signaling pathways. RCC is characterized by a constant state of hypoxia in non-hypoxic conditions due to *VHL* loss and consequent accumulation of HIF. This accumulation leads to upregulation of different genes such as VEGF and TGF-α, which ultimately induces VEGFR activation mainly on endothelial cells and consequent angiogenesis stimulation and tumor cells EGFR activation leading to cell proliferation and cell survival. Ultimately, these findings suggest that VEGF and EGFR could represent good therapeutic targets in RCC. According to our *in vitro* results the combination of bevacizumab, a VEGF sequester, and erlotinib, an EGFR inhibitor, seem to have a greater effect on a RCC cell line treatment when compared with erlotinib or bevacizumab alone. However, clinical trials demonstrated that erlotinib alone or in combination with bevacizumab does not seem to represent a good therapeutic option in mRCC patients highlighting the need to study and evaluate potential treatment resistance mechanisms.

MiRNAs may be a way to unveil targeted therapies resistance since they regulate gene expression of different proteins involved in crucial cellular processes. MiRNA-210, an HIF regulated miRNA, seems to be an important mediator of erlotinib resistance in RCC. Regarding our *in vitro* results, intracellular and extracellular miRNA-210 levels were upregulated in an erlotinib-resistant RCC cell and by targeting MNT seems to increase c-Myc-Max interaction in the G1 phase of the cell cycle. C-myc-Max heterodimer is an important mediator of cell cycle entry and progression since it binds to DNA and increases the expression of certain genes such as cyclins and CDKs. Taken together these results, miRNA-210 may represent a good biomarker for treatment response allowing a more personalized RCC patient´s treatment and also be seen as potential adjuvant therapy to erlotinib using a delivery model that allows miRNA stability and tumor-specific action. Further studies should be performed in G1 phase of the cell cycle in the erlotinib-resistant

RCC cell line and the parental RCC cell line. First, miRNA-210 mediated resistance mechanism should be further validated and replicated using a miRNA-210 inhibitor. Moreover, MNT-Max interaction should also be evaluated in order to verify if the increase in the c-Myc-Max interaction in the erlotinib-resistant RCC cell line is concomitantly related with a decrease in MNT-Max interaction. Ultimately, an evaluation of protein expression of genes regulated by c-Myc-Max heterodimer should also be performed, to study if the increase in c-Myc-Max interaction in the erlotinib-resistant RCC cell line as a functional consequence.

In vivo studies could also be performed to validate our findings and evaluate if miRNA-210 could be used as a circulating biomarker in erlotinib treatment response in RCC patients, and even if a miRNA-210 inhibitor could be considered as adjuvant therapy when using erlotinib.

7. References

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8. Attachments

Review

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Pharmacogenomics

miRNAs: mediators of ErbB family targeted therapy resistance

The ErbB/HER tyrosine kinase receptors family plays a key regulatory role in different cellular processes by activating several signaling pathways. In different tumor types, mutations or overexpression of the ErbB family members are a common feature, which led to the development of targeted therapies against this receptors. Although with this kind of treatment we are heading to a more personalized medicine, the development of acquired resistance is still an issue, therefore, several studies focused on discovering the mechanisms behind it. More recently, miRNAs have been described as important mediators of acquired resistance, specifically, acquired resistance to ErbB family targeted therapies. Ultimately, miRNA-based therapeutics using exosomes as a drug delivery model can revolutionize today's approach of cancer treatment.

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Background

miRNAs

miRNAs are a class of noncoding RNAs (19–25 nucleotides in length) that control gene expression by either degrading or blocking translation of mRNAs, a process that depends on the miRNA and the respective mRNA target degree of complementarity [1]. miRNA biogenesis is a multiphase process which initiates at the nucleus with the transcription of a primary RNA, the pri-miRNA, by RNA polymerase II [2]. Following transcription, pri-miRNA is processed by Drosha, a RNase III endonuclease, alongside with cofactor DGCR8, creating a pre-miRNA [3]. This precursor ultimately is exported to the cytoplasm by Exportin 5 where is processed by another RNase, Dicer, leading to the production of a mature 22 base pairs miRNA duplex [2]. The mature miRNA enters the RNA-induced silencing complex (RISC), whose main components are TRBP, AGO1–4, GEMIN3 and GEMIN4 becoming, ultimately, a functional miRNA [4]. This complex binds to the target mRNA at 3′UTR region by complementarity leading to gene silencing [5].

A miRNA is not specific for a certain mRNA, it can regulate up to 100 different mRNAs and is also described that more than 10,000 mRNAs seem to be regulated by miRNAs [6]. Thus, changes in the miRNA processing and expression patterns could be associated with different pathologies, including cancer, suggesting that miRNAs are involved in many cellular function disorders, which includes carcinogenesis [7].

After the discovery of miRNA-15a and miRNA-16-1 as the first miRNAs with tumor suppressor functions in chronic lymphocytic leukemia in 2002, many miRNAs have been described as mediators of cancer-related signaling pathways, regulating proliferation, apoptosis, angiogenesis and even epithelial–mesenchymal transition (EMT), a key step for the metastatic process [8,9]. Since miRNAs are associated with different biological processes, they have been described

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to act as oncogenes, tumor suppressors or even modulators of cancer stem cells and metastasis formation. OncomiRNAs are, usually, overexpressed in cancer since they are known to downregulate tumor suppressor genes and tumor suppressor miRNAs are responsible for downregulating oncogenes, so are mostly underexpressed in malign neoplasms [10,11]. However, this dichotomous approach may have its limitations. For example, miRNA-17 is associated, in B-cell lymphoma, with faster tumor development, while it can suppress cancer growth by downregulating AIB1 expression in breast cancer [12,13]. Therefore, we have to take in consideration the fact that miRNAs may act in a tissue-specific manner such that a single miRNA can be either an oncomiRNA or a tumor suppressor miRNA.

Regarding the miRNAs role in cellular processes such as proliferation, apoptosis or angiogenesis, it is important to take into account the effect that changes in miRNA levels may have in treatment response, since targeted therapies are used for specific proteins and signal pathways related with this biological processes [8,9].

Cancer treatment

Chemotherapy, along with surgery and radiotherapy, has been a crucial approach for cancer treatment. These different types of treatment can be used alone or in different combinations, and either simultaneously or sequentially. However, chemotherapy-/radiotherapy-induced cell damage occurs preferentially but not exclusively in cancer cells, causing many side-effects [14]. For this reason, nowadays, a big effort is being made in order to improve a personalized medicine that focus on the discovery and development of molecular targeted drugs that take advantage of genetic addictions, dependencies and vulnerabilities of cancer cells. This type of methodology would be more specific than previous described approaches by minimizing side effects on normal cells [15]. Additionally, different high-throughput technologies, still under development, like genome sequencing and various kinds of microarrays, will allow the knowledge of the genetic, epigenetic and proteomic background, from each individual and tumor, which ultimately will lead to a more personalized treatment [16].

The concept of targeting a pathogenic driver abnormality using a small molecule was first validated in 1988 by the successful treatment of patients with acute promyelocytic leukemia harboring translocations in the *RARα* gene with all-trans retinoic acid [17]. Additionally, the use of imatinib, a BCR-ABL inhibitor, as a chronic myeloid leukemia treatment in 1996, marked the era of the design of small therapeutic molecules applied in cancer treatment [18]. The 5-year estimated overall survival rate for patients with this malignancy, characterized by the BCR-ABL translocation, was 89% when imatinib was used as initial therapy [19].

Even with all this development and improvement in cancer therapy, resistance to treatment still exists. Therapy failure is often due to development of drug resistance that may be inherent in a subpopulation of heterogeneous cancer cells or acquired subsequent to treatment [20]. A well-characterized resistance mechanism is related with the activity of ABC transporters. ABC transporters are transmembrane proteins responsible for the transport of a wide variety of substrates across cellular membranes, including hydrophobic drugs and antibiotic [21,22]. Overexpression of these proteins can be associated with reduced drug uptake, increased drug efflux and lead to lower drug efficacy and possibly to acquire drug resistance due to low drug levels in the cytoplasm [22]. The major members of the ABC transporters associated with multidrug resistance in cancer cells are ABCB1/MDR1, ABCCs (MRPs) and ABCG2 (BCRP/MXR/ABCP) [23]. However, several ABC transporters have been identified as transporters of cancer chemotherapeutics agents, acquired chemotherapy drug resistance can occur at many levels, modulated either by genetic or epigenetic factors. In fact, recent data demonstrate that the activity of certain miRNAs might be altered in order to achieve resistance to chemotherapy [24]. In the same line of thought, miRNAs can be as well linked to acquired resistance in molecular targeted therapy in several malignancy treatments. Latest evidences support this idea as it will be described next.

This review focus in the role of miRNAs as mediators of acquired resistance in ErbB family targeted therapies since this family plays a key regulatory role in nearly every aspect of cell biology. The ErbB/HER family contains four tyrosine kinase receptors, the epidermal growth factor receptor (EGFR/ErbB/Her1), Her2 (Neu, ErbB2), Her3 (ErbB3) and Her4 (ErbB4) [25]. Different factors as the identity of the ligand and oligomer composition of the receptor determine the specificity and potency of intracellular signals [26]. Downstream ErbB signaling includes phosphatidylinositol 3-kinase/Akt (PKB) pathway, the Ras/Raf/MEK/ERK 1/2 pathway and the phospholipase C (PLC-γ) pathway [27]. All of these pathways are interconnected and overlapping [26]. Overall, they regulate apoptosis, cell cycle progression, cytoskeletal rearrangement, differentiation, development, immune response, nervous system function and transcription [28]. Mutations or increased expression of ErbB family members occur in several malignancies [26,29]. For instance, ErbB1 overexpression occurs in head and neck, breast, bladder, prostate, kidney,

non-small-cell lung cancer (NSCLC) and glioma tumors, while mutations leading to a constitutively active receptor occur in glioma, lung, ovary and breast cancer. Overexpression of ErbB2 is frequent in breast, lung, pancreatic, colon, esophagus, endometrium and cervix cancer, whereas ErbB3 is overexpressed in oral squamous cell cancer [26,30].

Several targeted drugs have been developed against these protein kinases, however, cancers submitted to targeted therapy eventually become resistant [31,32]. miRNAs may be a way not only to unveil resistance mechanisms (Table 1) but also, if used as a treatment option, to overcome targeted therapy limitations.

miRNAs & targeted therapy resistance

ErbB targeted therapies resistance in cancer

Head & neck cancer

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common form of cancer worldwide with 650,000 new cases each year [45]. Palliative chemotherapy and the EGFR inhibitor, cetuximab, constitute the backbone of treatment for patients with HNSCC [46]. However, many patients with HNSCC tumors do not respond to EGFR-targeting therapies [47].

Hatakeyama and coworkers demonstrated that one potential mechanism of acquired resistance to cetuximab in HNSCC involves the increased expression of HB-EGF that is regulated by miRNA-212. HB-EGF is known to bind both EGFR and HER4 and to induce EMT, enhance metastasis and modulate chemotherapy resistance [48–50]. miRNA-212 showed a 27-fold decrease in 1Cc8 cetuximab-resistant cell line relative to SCC1 cetuximab-sensitive cell line. Expression levels of HB-EGF and miRNA-212 were also examined in 32 additional HNSCC cell lines and keratinocyte cell line, demonstrating the inverse correlation of this two parameters. Increased expression of HB-EGF regulated by miRNA-212 and activation of receptor kinases other than EGFR, like HER3 and MET, and subsequent activation of AKT, were observed in 1Cc8 cell line, and may play an important role in acquired resistance to cetuximab [33].

Lung cancer

Lung cancer is the leading cause of cancer related death worldwide [51]. Of all lung cancer cases, approximately 80–85% correspond to NSCLC [52]. One of the main issues regarding the therapeutic approach using chemotherapy or EGFR-tyrosine kinase inhibitors (TKIs) in NSCLC is the acquired resistance that develops short after treatment [35,53]. In fact, the role of miRNAs, more specifically miRNA-21, has already been reported in NSCLC as a modulator of chemotherapy sensitivity [54]. Additionally, Shen *et al.* stated that this same miRNA correlated with PTEN levels (one of its most important targets) modulates gefitinib resistance in the same tumor model [55]. They ana-

Table 1. Summary of the miRNAs involved in the acquired resistance to ErbB family targeted therapies by cancer type.

Cancer	Targeted Therapy	miRNA involved	Ref.
Head and neck cancer	Cetuximab	↓ miRNA-212	[33]
Lung cancer	Erlotinib	↑ miRNA-200 family	[34]
	Gefitinib	↑ miRNA-21	[35]
		↑ miRNA-30c, ↓ miRNA-103, ↓ miRNA-203, ↑ miRNA-221 and ↑ miRNA-222	[36]
		↑ miRNA-214	[37]
		↑ miRNA-374a and ↓ miRNA-548b	[38]
Gastric cancer	Trastuzumab	↑ miRNA-21	[39]
Breast cancer	Trastuzumab	↑ miRNA-21	[40]
		↑ miRNA-221	[41]
		↓ miRNA-375	[42]
	Lapatinib	↓ miRNA-630	[43]
	Neratinib		
	Afatinib		
Coloretal cancer	Cetuximab	↓ miRNA-let7b, ↓ miRNA-let7e and ↑ miRNA-17	[44]

↑: Upregulation; ↓: Downregulation.

lyzed the expression of miRNA-21 and PTEN protein in tumor tissues from NSCLC patients, comparing cancer tumor specimens with adjacent normal tissues. A significantly higher expression of miRNA-21 and a reduction in PTEN protein levels was found in tumor tissues, demonstrating a negative correlation. High miRNA-21/low PTEN expression levels indicated a poor TKI clinical response and shorter overall survival in NSCLC patients. In order to test the effect of high miRNA-21/low PTEN expression on modulation of TKI sensitivity, a PC-9 TKI-sensitive cell line and a gefitinib-resistant cell line PC-9/GR were used. *In vitro* assays showed that miRNA-21 was upregulated concomitantly to downregulation of PTEN in PC-9/GR cells. Moreover, overexpression of miRNA-21 significantly decreased gefitinib sensitivity by down-regulating PTEN expression and activating AKT and ERK pathways in PC-9 cells. Whereas, miRNA-21 knockdown dramatically restored gefitinib sensitivity of PC-9/GR cells by up-regulation of PTEN expression and inactivation of AKT and ERK pathways, both *in vivo* and *in vitro* [56].

Another study performed by Izumchenko and coworkers demonstrated that TGFβ–miRNA200–MIG6 pathway coordinates the EMT-associated kinase switch that induces resistance to EGFR inhibitors [34]. They evaluated pairs of cancer cell lines with wild-type *EGFR* that were either sensitive (epithelial-like) or resistant (mesenchymal-like) to erlotinib, an EGFR TKI. Treatment of erlotinib-sensitive cell lines with TGFβ resulted in complete EMT. Cells with induced mesenchymal phenotype, had both total EGFR and phospho-EGFR reduced and elevated expression of MIG6, acquiring a relative resistance to erlotinib, associated with a significant increase in AKT activity, due to higher levels of phospho IGFR, PDGFR, FGFR and FAK kinases [36,57]. Concurrently, expression levels of miRNA200 family decreased significantly. During TGFβ-mediated EMT, inhibition of the miRNAs 200 family results in upregulated expression of the MIG6, a negative regulator of EGFR. The MIG6-mediated reduction of EGFR occurs concomitantly with a TGFβ-induced EMT-associated kinase switch of tumor cells to an AKT-activated EGFR-independent state. The expression levels MIG6 (mRNA)/miRNA200 ratio were inversely correlated with EMT and resistance to erlotinib, in both *in vitro* and *in vivo* models. Demonstrating that TGF-β–miRNA200–MIG6 network orchestrates the EMT-associated kinase switch that induces resistance to EGFR inhibitors [34].

Studies in NSCLC also revealed an involvement of MET oncogene in TKIs resistance [37,58]. Garofalo and coworkers demonstrated that MET and EGFR-related miRNAs had a significant role in gefitinib resistance on NSCLC cell lines and *in vivo* models. NSCLC gefitinib-resistant cell lines, Calu-1 and A549, did not revealed an expected miRNA-30b-c and miRNA-221/-222 down-regulation and consequent increase in BIM and APAF-1 protein levels after treatment. miRNA-30b-c and miRNA-221/-222 knockdown increased gefitinib sensitivity in resistant and sensitive gefitinib cell lines indicating that these miRNAs are important modulators of TKI resistance. Results from the same paper also show that MET overexpression controls gefitinib resistance through activation of the AKT/ERKs pathway, mediated at least in part by the miRNA-103 and -203 downregulation since an induced expression of these miRNAs increases Calu-1 cells gefitinib sensitivity. Additionally, Dicer knockdown reduced gefitinib resistance and also migration and the expression of mesenchymal markers. Since miRNA-103 targets Dicer, these results may suggest that this miRNA could be involved in the EMT process through Dicer down-regulation. Ultimately, all these results were supported by *in vivo* studies since miRNA-103 and miRNA-203 overexpression or miRNA-221 and -30c knockdown resulted in tumor growth inhibition and increased sensitivity to gefitinib in nude mice after treatment [59].

miRNA-214 has also been described as a gefitinib resistance mediator [60]. After exposure to increasing concentrations of gefitinib, studies performed by Y-S Wang and coworkers in the resistant clone of a lung adenocarcinoma cell line, HCC827/GR, revealed an overexpression of miRNA-214. The upregulation of this miRNA leads to a PTEN down-regulation, which is involved in PI3K–AKT pathway [57]. PTEN protein dephosphorylates PI3K, that mediates activation of AKT, ultimately leading to an inactivation of this pathway [38]. So, miRNA-214 mediates gefitinib resistance in this model by activating PI3K/AKT pathway, which has been described to confer resistance to EGFR-TKI by overcoming the EGFR blocking in previous studies [61]. Finally, miRNA-214 knockdown led to gefitinib sensitivity in HCC827/GR [60].

Additionally, other studies performed by Wang *et al.* also revealed a gefitinib resistance in NSCLC cell lines and *in vivo* models, but this time mediated by Axl-altered miRNAs. Findings of the involvement of Axl kinase in acquired resistance to TKIs in this tumor model were prior to this article but Wang and his fellow workers proposed the involvement of the miRNA-374a and miRNA-548b in this resistance [62]. Analysis of the miRNA expression profile was performed in a generated gefitinib-resistant cell line, HCC827-Gef, in Calu1 cell line, which is resistant to TKI, and in tumor samples. Results revealed a relationship between Axl overexpression and the overexpression of miRNA-374a

and downregulation of miRNA-548b not only in the gefitinib-resistant cell lines but also in tumor samples. Knockdown of miRNA-374a and upregulation of miRNA-548b increased the sensitivity to gefitinib in gefitinib-resistant cell lines revealing their importance in this mechanism. Finally, results from the same authors also showed that miRNA-374a and miRNA-548b not only have a role in gefitinib sensitivity and gefitinib-induced apoptosis but also essential roles in cell cycle arrest, EMT, migration and tumorigenesis of gefitinib-resistant lung cancer cells *in vitro* and *in vivo* by targeting WNT5A and CCNB1, respectively [63].

Gastric cancer

Gastric cancer (GC) is the fourth most commonly diagnosed cancer and the second most common cause of cancer related death worldwide [39]. Results from a recent large-scale Phase III study demonstrated that trastuzumab combined with standard chemotherapy provided a significant survival advantaged compared with chemotherapy alone in advanced HER2-positive GC [64]. Even though trastuzumab can prolong the survival of patients with HER2-positive GC, most of them end up developing resistance, highlighting the importance in clarifying the mechanisms behind this event [65].

Eto and coworkers described that miRNA-21/PTEN pathway regulated the sensitivity of HER2-positive GC cell lines to trastuzumab through modulation of apoptosis. On one hand, they were able to demonstrate that overexpression of miRNA-21 not only downregulated PTEN expression but also increased AKT phosphorylation, however, not affecting HER2 expression. On the other hand, suppression of miRNA-21 increased PTEN expression and downregulated AKT phosphorylation, still not affecting HER2 expression. In addition, overexpression of miRNA-21 decreased GC cells sensitivity to trastuzumab by suppression of apoptosis; whereas suppression of miRNA-21 expression restored trastuzumab sensitivity of GC cells. These findings suggest that miRNA-21/PTEN pathway may be crucial to trastuzumab acquired resistance mechanism in GC [66].

Breast cancer

Breast cancer remains the most frequently diagnosed malignancy and the primary cause of cancer-related death in women globally [51]. HER2 overexpression occurs in 10–34% of invasive breast cancers [41]. HER2-positive breast cancers are associated with more aggressive tumor phenotypes and often acquired resistance to therapy [67,68]. Additionally, the downregulation of key miRNA processing enzymes, such as Drosha and Dicer, have been associated with the outcome, progression and recurrence of breast cancer. In fact, it was shown that Dicer is an independent predictor of recurrence in the HER2-positive subtype [40,42,69]. Ye and coworkers showed that in a HER2-positive breast cancer cell line, SK-BR-3, miRNA-221 knockdown led to a significant decrease of surviving cells in the presence of trastuzumab, while overexpression of the pre-miRNA in question led to the opposite result. Trastuzumab resistance in this tumor model seems to be mediated by tumor suppressor PTEN, since a miRNA-221 overexpression leads to a PTEN downregulation [43,70].

The activation of IGF1R, an alternative growth factor receptor, represents a common feature of trastuzumab-refractory cells [71]. However, the underlying mechanism remained unclear until very recently, when Xing-Ming and coworkers demonstrated that epigenetic silencing of miRNA-375 induces trastuzumab resistance in HER2-positive breast cancer by targeting IGF1R [72]. Their findings revealed that miRNA-375 targeted IGF1R and was downregulated in trastuzumab-resistant HER2-positive breast cancer cells. While overexpression of miRNA-375 restored trastuzumab sensitivity in cells, inhibition of miRNA-375 induced trastuzumab resistance in HER2-positive breast cancer cells. They also showed that regulation of miRNA-375 expression was epigenetic since inhibition of DNA methylation and histone deacetylation restored the expression of miRNA-375 in trastuzumab-resistant cells. Additionally, they found a negative correlation between the levels of miRNA-375 and IGF1R in breast cancer tissue samples. Lastly, epigenetic silencing of miRNA-375 causes IGF1R upregulation, which at least partially explains the mechanism of trastuzumab resistance in breast cancer cells [72].

A similar work was performed by Gong *et al.* which consisted in *in vitro* experiments and *in vivo* analysis of HER2-positive breast cancers. HER2-positive-trastuzumab-resistant cell lines were obtained by doing cell cultures in the presence of low-dose trastuzumab. miRNA analysis concluded that miRNA-21 was overexpressed in all resistant cell lines in comparison with parental ones. On one hand, knockdown of this miRNA resensitized the trastuzumab resistant breast cancer cells and its effects in proliferation and cell cycle. On the other hand, ectopic expression of miRNA-21 led to trastuzumab resistance in parental cell lines. Trastuzumab resistance mediated by miRNA-21 appears to be via tumor suppressor PTEN, since overexpression of this miRNA led to lower PTEN protein levels. Retrieving PTEN expression in resistant breast cancer cells restored trastuzumab activity, since this targeted therapy enhances PTEN phosphatase activity leading to AKT dephosphorylation [73]. *In vivo* studies were also performed and similar results were obtained leading to

the conclusion that miRNA-21 overexpression in tumor xenografts results in resistance to trastuzumab by inhibiting PTEN expression. Finally, the clinical significance of these findings was evaluated by examining primary breast cancers from patients receiving trastuzumab therapy. miRNA-21 expression in breast cancer cells was reversely correlated with PTEN expression, and in line with a miRNA-21 upregulation, PTEN expression was lower in trastuzumab-resistant tumors [44].

Also, recent studies performed by Corcoran *et al.* in breast cancer cell lines revealed an involvement of miRNA-630 in resistance to HER-targeting drugs such as lapatinib, neratinib and afatinib [74]. Lapatinib-resistant SKBR3 and HCC1954 cells and neratinib-resistant HCC1954 cells showed a decrease in intra and extracellular levels of the miRNA-630 when compared with the parental cell lines. Transfection of miRNA-630 mimic to resistant cell lines enhanced the

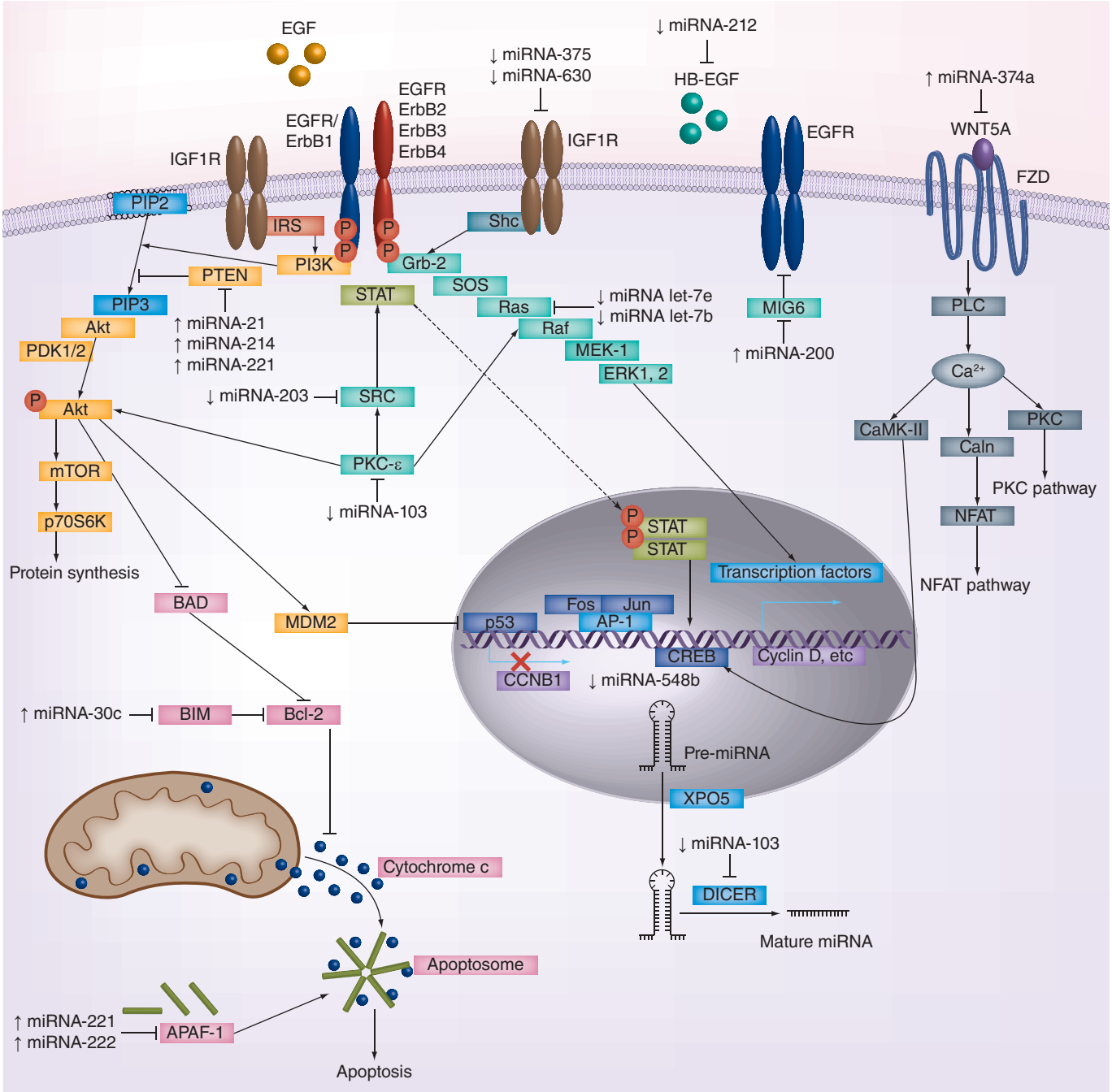

Figure 1. Schematic representation of the miRNAs involved in ErbB targeted therapy acquired resistance and their targets in the multiple cell signaling pathways.

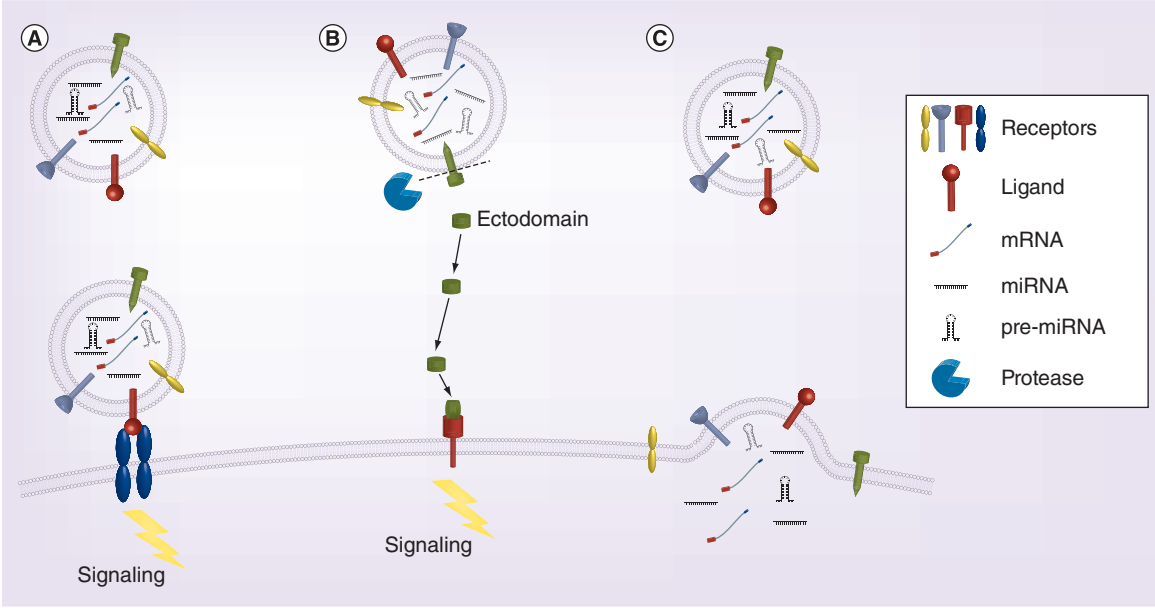

Figure 2. Possible mechanisms of exosome-target cell interaction. (A) Interaction between exosomal membrane proteins and target cell receptors leads to intracellular signaling activation – juxtacrine fashion; **(B)** Interaction between an ectodomain, formed after protease cleavage of exosomal membrane proteins, and target cell receptors also leads to signaling pathways activation; **(C)** Fusion of the exosome with the recipient cell membrane and content release to the intracellular space in a nonselective manner.
Adapted with permission from [75].

antiproliferative effects of all the drugs in study. The miRNA-630 dependent resistance mechanism seems to be related, not only with the increase of IGF1R levels, a target of miRNA-630, but also with the increase of EGFR and HER2 levels, targets of the drugs in study. The levels of the phosphorylated form of these proteins are also increased when miRNA-630 inhibition is performed in breast cancer cell lines. Finally, Corcoran *et al.* also proved that inhibition of the miRNA-630 in breast age-matched cancer cells was associated with increased motility, migration, invasion and resistance to anoikis [74].

Colorectal cancer

Colorectal cancer (CRC) is the third most common cancer worldwide and the fourth most common cause of death, affecting men and women almost equally [75]. The use of monoclonal antibodies against EGFR, such as cetuximab and panitumumab, are a common pharmacologic approach in CRC treatment [76]. Besides the discovery that acquired *KRAS* mutations are a good predictive marker of cetuximab and panitumumab resistance in CRC, it is still unclear why certain patients respond to therapy and others do not [76,77].

Ragusa and coworkers made an expression profile of 667 miRNAs in two human colorectal cancer cell lines, one cetuximab sensitive (Caco-2) and other cetuximab resistant (HCT-116). They identified a group of miRNAs differentially expressed and tested them in CRC patients. miRNAs let-7b and let-7e were downregulated in HCT-116 after cetuximab treatment, in which signaling downstream of KRAS remains activated. Let-7 family members are known to target KRAS, so their downregulation could be a mechanism that contributes to cetuximab resistance. Additionally, miRNA-17* (a CRC marker) is up-regulated in the resistant-cell line and downregulated in the sensitive one, after cetuximab treatment [78]. Taken together, miRNA let-7b, let-7e and 17* could be considered as candidate molecular markers of cetuximab resistance [79].

Regarding all this results, we observe that, in some cases, different miRNAs are involved in different acquired resistance mechanisms for the same drug and in the same tumor model. miRNAs are not specific for a single mRNA, moreover mRNAs and consequently proteins are not regulated by only one miRNA [6]. Additionally, signaling pathways related with ErbB family overlap [26]. With this being said, it is more likely that an acquired resistance to a targeted therapy would be mediated by a network of miRNAs rather than a single one, targeting multiple steps of different pathways (Figure 1). However, since miRNAs play a major role in targeted therapy resistance, more specifically, resistance to ErbB family targeted therapies, we could consider them as therapeutic options. The use of miRNAs mimics or inhibitors, when drug resistance is due to an under-expression or overexpression, respectively, of the miRNA in question, should be consid-

ered as adjuvant therapy to drugs such as cetuximab, erlotinib, gefitinib and others.

The main issue regarding the use of miRNAs as therapeutic options is the specificity of the delivery *in vivo*. In other words, a direct delivery to the tumor, without affecting other body parts. Many studies have been made which led to huge improvements in this area and this introduces our next topic, exosomes.

Exosomes: a potential drug delivery model in EGFR-overexpressing tumors

Exosomes are membrane-bound vesicles of 40–100 nm in diameter present in almost all biological fluids [80]. They are released from most cell types, including cancer cells, into the extracellular space after fusion with the plasma membrane [81]. This type of extracellular membrane vesicles are enriched in cholesterol, sphingomyelin and ceramide as well as lipid raft associated proteins [81,82]. As a consequence of their origin, nearly all exosomes, independently of the cell type from which they originate, contain similar composition. However, the exosomal lumen, which is in part composed by mRNAs, miRNAs and other noncoding RNAs is determined by the cell type which produced the exosomes [83]. In the last years, many authors reported differences in miRNA content when comparing exosomes from normal individuals with cancer patients [84,85]. Since exosomes are released and are able to circulate in most biological fluids, they can interact with neighboring or distant cells and ultimately lead to the modulation of the recipient cells [86]. There are three main possible mechanisms of intracellular communication by exosomes. First, in a juxtacrine fashion, exosomal membrane proteins can interact with receptors in a target cell and activate different signaling pathways. Second, proteases in the extracellular space can cleave exosomal membrane proteins, leading to a cleaved fragment-target cell surface receptor interaction. This mechanism also activates intracellular signaling. Finally, exosomes can fuse with the target cell membrane and release their contents, such as mRNAs and miRNAs, which can alter gene expression and protein translation of the recipient cell (Figure 2) [81]. The ability of exosomes to interact and modulate target cells and also their high stability in circulation makes them good candidates to *in vivo* delivery of different molecules, such as miRNA mimics or inhibitors, which would allow us to surpass targeted therapies resistance mediated by miRNAs [87]. In fact, exosomes have already been used in *in vivo* models with different purposes and in a wide-range of diseases [88]. More specifically, in cancer Phase I clinical trials, exosomes are being used to, either increase innate and adaptive immune responses against the tumor, or deliver therapeutic agents in a cancer-specific way [88]. Related with the exosomes high stability and ability of travelling in biological fluids, a Phase I clinical trial is investigating the ability of plant exosomes to deliver curcumin to normal and colon cancer tissue, since previous studies demonstrated that curcumin has a strong inhibitory effect on the growth of colon cancer cell lines by mediating signal transduction [89,90].

Regarding the focus of our review article, more recently, Ohno *et al.* showed that exosomes could be used as drug delivery carriers in an EGFR-overexpressing cancer model. They used modified exosomes, with GE11 peptide in their membranes, to specifically deliver exosomal content to EGFR-expressing breast cancer cells. GE11 peptide binds to EGFR and is markedly less mitogenic than EGF. Also, in the same paper, efficient *in vivo* delivery of let-7a, a miRNA that functions as a tumor suppressor, was achieved by loading it to GE11^{+} exosomes and by injecting the modified exosomes intravenously in RAG2$^{-/-}$ mice, that were submitted, previously, to breast cancer cells transplantation [91]. This treatment suppressed tumor growth and no major organ damage was detected in the injected mice [92].

Although an exosome-based drug delivery model seems promising, many challenges still rise and have to be overcome. One of the main issues is the inexistence of a standard technique to isolate and purify exosomes. Usually, ultracentrifugation is used to obtain exosomes, however this technique has some limitations since it leads to low production yield and contamination with protein aggregates and cellular debris, which may affect the quality of these nanovesicles [93]. Another issue is the lack of biochemically well-characterized exosomes and the fact that the exosome protein content varies depending on the cells that produced them [83,93]. These facts could determine the safety and effectiveness of an exosome-based treatment since certain molecules, such as MHC class I or II, could trigger a host immune response and eliminate these vesicles. Finally, the method of drug loading to exosomes should be optimized since the efficiency is relatively low [93]. Once these issues are surpassed, exosomes could become a novel therapeutic approach not only in cancer but in many other diseases allowing a more specific and potent drug effect.

Conclusion

miRNAs are an important epigenetic mechanism of acquired resistance to targeted therapies by cancer cells. Despite great findings lately, this subject still needs further research in order to completely understand the mechanisms underlying acquired resistance of different drugs in a wider range of tumor types.

Executive summary

miRNAs

- miRNAs are a class of noncoding RNAs that control gene expression by either degrading or blocking translation of mRNAs.
- A miRNA is not specific for a certain mRNA, it can regulate up to 100 different mRNAs and is also described that more than 10,000 mRNAs seem to be regulated by miRNAs.
- Changes in miRNA levels may have an effect in treatment response, since targeted therapies are used for specific proteins and signal pathways related with biological processes, which are regulated by miRNAs.

Cancer treatment

- Chemotherapy and radiotherapy target, but not exclusively, cancer cells, causing several side-effects. Target therapy emerged in order to minimize those side-effects.
- Despite all the latest developments and improvements in cancer therapy, resistance to treatment still exists.
- Acquired chemotherapy drug resistance can be modulated by genetic and/or epigenetic factors. In fact, recent data demonstrate that certain miRNAs activity might be altered in order to achieve chemotherapy resistance.
- The ErbB/HER receptors family regulate apoptosis, cell cycle progression, cytoskeletal rearrangement and many other biological processes. Mutations or increased expression of ErbB family members occur in several malignancies, thus different ErbB/HER targeted therapies have been developed against this family of receptors.
- miRNAs may be a way not only to unveil resistance mechanisms but also, if used as a treatment option, to overcome targeted therapy limitations.

miRNAs & targeted therapy resistance

- One potential mechanism of acquired resistance to cetuximab in head and neck squamous cell carcinoma involves the increased expression of HB-EGF due to a decrease in miRNA-212 expression. HB-EGF promotes cetuximab resistance since it activates receptor kinases other than EGFR, like HER3 and MET.
- High expression levels of miRNA-21 are associated with gefitinib resistance in non-small-cell lung cancer (NSCLC) cell lines and also with trastuzumab resistance in HER2 positive gastric and breast cancer cell lines. The upregulation of this miRNA leads to a PTEN downregulation, which is involved in PI3K-AKT pathway. PTEN protein dephosphorylates PI3K that mediates activation of AKT, ultimately leading to AKT pathway inactivation.
- miRNA-214 and miRNA-221 overexpression are associated with gefitinib resistance in a lung adenocarcinoma cell line and trastuzumab resistance in a breast cancer cell line, respectively. Since PTEN is a target of these miRNAs, the resistance mechanism is also based in a PI3K/AKT pathway activation.
- In different lung cancer cell lines it has been demonstrated that TGF-β–miRNA200–MIG6 pathway coordinates the EMT-associated kinase switch that induces resistance to erlotinib.
- Garofalo and coworkers demonstrated that changes in MET and EGFR-related miRNAs levels, more specifically, miRNA-30c, miRNA-103, miRNA-203, miRNA-221 and miRNA-222 levels, had a significant role in gefitinib resistance on NSCLC cell lines and *in vivo* models.
- The overexpression of miRNA-374a and the downregulation of miRNA-548b have been correlated with Axl kinase overexpression that is involved in acquired resistance to gefitinib in NSCLC.
- miRNA-375 downregulation by epigenetic silencing causes IGF1R upregulation, which leads to trastuzumab resistance in HER2-positive breast cancer.
- In breast cancer cell lines, miRNA-630 downregulation is associated with resistance to HER-targeting drugs such as lapatinib, neratinib and afatinib. The miRNA-630 dependent resistance mechanism seems to be related, not only with the increase of IGF1R levels, a target of miRNA-630, but also with the increase of EGFR and HER2 levels, targets of the drugs in study.
- Let-7 family members are known to target KRAS, so let-7b and let7-e downregulation could be a mechanism that contributes to cetuximab resistance in colorectal cancer.

Exosomes: a potential drug delivery model in EGFR-overexpressing tumors

- Exosomes are membrane-bound vesicles released from most cell types, including cancer cells.
- The exosomal lumen, which is in part composed by mRNAs, miRNAs and other noncoding RNAs is determined by the cell type which produced the exosomes.
- Exosomes can interact with surroundings or distant cells and ultimately lead to the modulation of the recipient cells, since they are able to circulate in most biological fluids.
- The ability of exosomes to interact and modulate target cells and also their high stability in circulation makes them good candidates to *in vivo* delivery of different molecules, such as miRNA mimics or inhibitors.
- In Phase I clinical trials, exosomes are being used to, either increase innate and adaptive immune responses against the tumor, or deliver therapeutic agents in a cancer-specific way.

Ultimately, these findings would allow a better understanding of tumor biology and therapy response, enforcing the development of a treatment even more personalized and efficient. In this review, we have highlighted miRNAs and their role in the development of acquired resistance to ErbB family targeted therapies in different cancer models.

Future perspective

In the future, miRNAs could be used as biomarkers for treatment response, through an expression profile, and also as therapeutic options. Modified exosomes carrying antitumor miRNAs are a promising drug delivery model that can revolutionize today's approach of cancer treatment.

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